EXHIBIT A

TGF-β signalling from cell membrane to nucleus through SMAD proteins

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The recent identification of the SMAD family of signal transducer proteins has unravelled the mechanisms by which transforming growth factor-β (TGF-β) signals from the cell membrane to the nucleus. Pathway-restricted SMADs are phosphorylated by specific cell-surface receptors that have serine/threonine kinase activity, then they oligomerize with the common mediator Smad4 and translocate to the nucleus where they direct transcription to effect the cell's response to TGF-β. Inhibitory SMADs have been identified that block the activation of these pathway-restricted SMADs.

TGF-β1 is the prototype of a large family of cytokines that includes the TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs) and Müllerian-inhibiting substance (reviewed in ref. 1) (Table 1). Members of the TGF-β family exert a wide range of biological effects on a large variety of cell types, for example they regulate cell growth, differentiation, matrix production and apoptosis. Many of them have important functions during embryonal development in pattern formation and tissue specification; in the adult they are involved in processes such as tissue repair and modulation of the immune system.

Here we discuss recent breakthroughs in our understanding of the mechanisms used by members of the TGF-β family to elicit their effects on target cells, focusing on the pivotal role of SMAD proteins in relaying signals from cell-surface receptors to the nucleus.

Signalling through receptor complexes

TGF-β family members initiate their cellular action by binding to receptors with intrinsic serine/threonine kinase activity. This receptor family consists of two subfamilies, type I and type II receptors, which are structurally similar, with small cysteine-rich extracellular regions and intracellular parts consisting mainly of the kinase domains. Type I receptors, but not type II receptors, have a region rich in glycine and serine residues (GS domain) in the juxtamembrane domain. Each member of the TGF-β superfamily binds to a characteristic combination of type I and type II receptors (Table 1), both of which are needed for signalling.

Studies of the receptors for TGF-β have provided a model for the activation of these serine/threonine kinase receptor complexes[2]. TGF-β1 first binds to the type II receptor (TβR-II), which occurs in the cell membrane in an oligomeric form with activated kinase[3,4]. Then, the TGF-β type I receptor (TβR-I), which may also occur in an oligomeric form[5] and cannot bind TGF-β in the absence of TβR-II, is recruited into the complex; TβR-II phosphorylates TβR-I in the GS domain to activate it. The assembly of the receptor complex is triggered by ligand binding, but the complex is also stabilized by direct interaction between the cytoplasmic parts of the receptors[6]. The model[2] predicts that the type II and type I receptors act in sequence, which is supported by the finding that a constitutively active TβR-I (Thr 204 replaced with an aspartate residue) is able to exert TGF-β signals in the absence of TβR-II (ref. 7). It is likely that other serine/threonine kinase receptor complexes are also activated by a similar mechanism[8,9], although some variations on the theme have been noted. One of the TGF-β isoforms (TGF-β2) binds only with low affinity to TβR-II and requires the cooperation with TβR-I or betaglycan, an accessory transmembrane proteoglycan, for high-affinity binding[10]. Moreover, BMPs bind

Table 1 TGF-β family members, their receptors and signalling molecules

Subfamily	TGF-β	Activin	BMP
Examples of ligands	TGF-β1 TGF-β2 TGF-β3	Activin A	BMP-2 BMP-4 BMP-7/OP-1
Type II receptors	TβR-II	ActR-II ActR-IIB	BMPR-II ActR-II ActR-IIB
Type I receptors	TβR-I	ActR-I? ActR-IB	BMPR-IA BMPR-IB ActR-I
Pathway-restricted SMADs	Smad2 Smad3	Smad2 Smad3	Smad1 Smad5 Smad9?
Common-partner SMAD	Smad4	Smad4	Smad4
Inhibitory SMADs	Smad6 Smad7	Smad6 Smad7	Smad6 Smad7
Responses	Inhibition of mitogenicity Induction of extracellular matrix	Induction of dorsal mesoderm Induction of erythroid differentiation Induction of follicle-stimulating hormone release	Induction of ventral mesoderm Induction of cartilage and bone Induction of apoptosis

The three best-characterized vertebrate TGF-β subfamilies are listed.

with low affinity to BMP type I or type II receptors individually, and with high affinity only when the two BMP receptor types are presented together[11–13].

Analysis of ^{125}I-labelled TGF-β1 crosslinked to its receptors has suggested that the signalling complex is a heterotetramer consisting of two TβR-I and two TβR-II molecules[14]. This conclusion is supported by experiments using chimaeric erythropoietin/TGF-β receptors which showed that both homodimerization of type I receptors and hetero-oligomerization with the type II receptor are needed for the antimitogenic effect. Moreover, studies of a series of signalling-defective TβR-I receptors revealed that a kinase-defective TβR-I can complement an activation-defective TβR-I, suggesting that the signalling complex consists of at least two TβR-I molecules[15].

In the receptor-activation model, TβR-I acts downstream of TβR-II for most, if not all, TGF-β-mediated responses, and the type I receptor thus determines the specificity of the intracellular signals[16]. A nine-amino-acid sequence between kinase subdomains IV and V of TβR-I, which diverges between the different type I receptors, is important for transduction of specific TGF-β signals[17] (Fig. 1).

Situations have been described where, as a result of a decrease in expression of TβR-II but not TβR-I, cells lose the antiproliferative response to TGF-β, whereas the matrix accumulation induced by TGF-β is retained[18,19]. Moreover, expression of a dominant-negative TβR-II was found to block the growth-inhibitory effect of TGF-β, but not the effect on extracellular matrix[19,20]. These observations are compatible with a more important role for TβR-II in the antiproliferative response than in the matrix response, but they do not necessarily contradict the sequential activation model in which activated TβR-I is required for both responses; different effects of TGF-β may occur at different threshold levels of stimulation, with the antiproliferative effect requiring a more efficient stimulus than the effect on matrix, for example.

An important step in receptor activation is phosphorylation of the tetrameric receptor complex. Phosphorylation sites in TβR-II and TβR-I have been mapped using wild-type and chimaeric receptors[21–23] (Fig. 1). Certain of the phosphorylation sites in TβR-II are important in modulating the signalling activity of the receptor; phosphorylation of Ser 213 and Ser 409 is required for TβR-II activity, whereas phosphorylation of Ser 416 inhibits TβR-II signalling[22]. Notably, TβR-II and the activin type IIB receptor autophosphorylate on tyrosine residues, as well as on serine and threonine residues, and so may function as dual-specificity kinases[23,24]. The importance of autophosphorylation on tyrosine residues remains to be determined.

TβR-I is phosphorylated by TβR-II at several residues in the GS domain, which leads to activation of the TβR-I kinase[22,21] (Fig. 1). It is possible that phosphorylation of TβR-II in the corresponding part of the juxtamembrane region[22] is also important for its activation. In addition, TβR-I is phosphorylated at Ser 165, which is located N-terminally of the GS domain. Mutation of Ser 165 gave a type I receptor with a more powerful signalling effect in growth inhibition and matrix accumulation, but a weaker apoptotic signal[21]. Thus phosphorylation of Ser 165 may modulate TGF-β signalling.

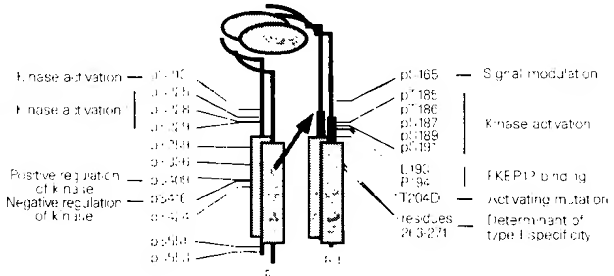

Figure 1 An activated TGF-β-receptor complex. The dimeric TGF-β molecule (light blue) binds to a heterotetrameric complex of two TβR-I and two TβR-II molecules. The GS domain (red) just upstream of the kinase domain (dark blue) of TβR-I is indicated. Known autophosphorylation sites in TβR-II and sites in TβR-I phosphorylated by TβR-II and their functional roles are indicated, as well as amino-acid residues involved in FKBP12 binding, activating mutation (T204D) and determination of TβR-I specificity in signalling (residues 26[illegible]–272). Assignments of phosphorylation sites are based on data in refs 21–23. The amino-acid numbers for sites in the TβR-I in ref. 21 differ by two owing to a mistake in the amino-acid numbering in this reference.

Downstream signalling mechanisms

Recent studies in the genetically accessible *Drosophila* and *Caenorhabditis elegans* have led to a breakthrough in our understanding of how signals are transduced from serine/threonine kinase receptors to the nucleus.

In *Drosophila*, the BMP-2/4 homologue Decapentaplegic (Dpp) acts by binding to the type II receptor Punt and to the type I receptors Thick veins and Saxophone. In a genetic screen for dominant enhancers of weak *dpp* alleles, *mothers against dpp* (*Mad*) and *Medea* were discovered[25,26]. Homozygous *Mad* mutants were found to have a phenotype similar to *dpp* mutants, with defects in midgut morphogenesis, imaginal disc development and embryonic dorsal–ventral patterning[26]. Evidence that Mad is a downstream component in the Dpp pathway came from the finding that *Mad* partially rescued the eye phenotype of dpp^{blk}[27], that Mad is required for the response to Dpp of the visceral mesoderm or endoderm[28], and that *Mad* mutations suppress dominant *thick veins* alleles[29]. There is also biochemical evidence that Mad functions downstream of Dpp receptors in *Drosophila*[30].

In *C. elegans*, *daf-1* and *daf-4* encode serine/threonine kinase receptors. *Daf-4* mutants are dauer-constitutive and smaller than wild-type; moreover, females are defective in egg-laying and males have fused tail rays. Screening for mutants with similar phenotypes revealed three genes, *sma-2*, *sma-3* and *sma-4*, which proved to be homologous to *Mad* of *Drosophila*[31]. As Sma-2 acts in the same cell as Daf-4 and *daf-4* is unable to rescue *sma-2* mutations, it was concluded that Sma molecules are involved in downstream signalling from the Daf-4 receptor.

SMADs are cytoplasmic mediators

At least nine genes homologous to *Mad* and *sma* have been identified in *Xenopus*, mouse and man, and shown to be components in signal transduction pathways downstream of serine/threonine kinase receptors (reviewed in ref. 1) (Fig. 2a). In an attempt to simplify the nomenclature, the designation *Smad* has been suggested for vertebrate homologues of *Sma* and *Mad*.

SMADs are molecules of relative molecular mass 42K–60K with two regions of homology at the amino and carboxy terminals, termed Mad-homology domains MH1 and MH2, respectively, which are connected with a proline-rich linker sequence (Fig. 2b). Recent work, which will be discussed below, suggests that in their inactive configurations, the MH1 and MH2 domains of SMADs make contact with each other: after activation by receptors, the molecules open up, form hetero-oligomeric complexes, and translocate to the nucleus where the transcription of target genes is affected.

Pathway-restricted SMADs. Different members of the SMAD family have different roles in signalling. Smad1, Smad2, Smad3 and possibly Smad5 interact with and become phosphorylated by specific type I serine/threonine kinase receptors and thereby act in a pathway-restricted fashion. An initial indication of a functional subspecialization among different SMADs came from the finding that *Xenopus* Smad1 (Xmad1) induces ventral mesoderm, a BMP

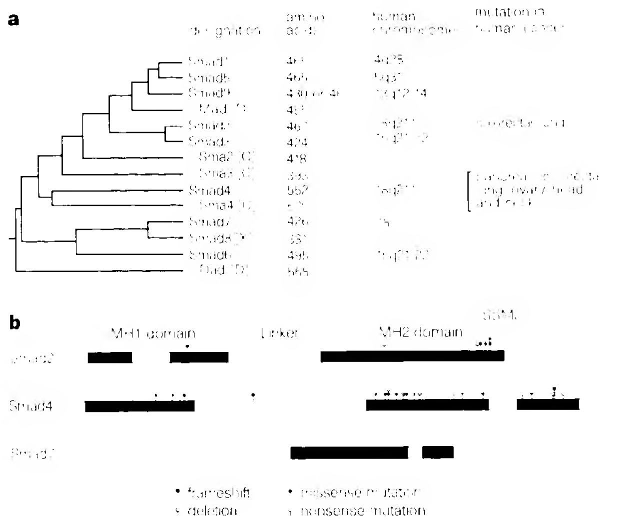

Figure 2 The SMAD family. a, A phylogenetic tree of human SMADs. Smad8 from *Xenopus* (X), Mad and Dad from *Drosophila* (D) and Sma from *C. elegans* (C) are shown. Alternative designations are as follows: Smad1 (Madr1, Xmad1, bsp1, DwfA, JV4-1), Smad2 (Madr2, Xmad2, JV18-1), Smad3 (hMad3, JV15-2), Smad4 (DPC4), Smad5 (Dwf-C, JV5-1), Smad6 (JV15-1) and Smad9 (MADH6). b, The sequences of representatives of pathway-restricted SMADs (Smad2), common-partner SMADs (Smad4) and inhibitory SMADs (Smad7) are shown to illustrate areas of homology between various types of SMAD molecules (black). Mutations in Smad2 and Smad4 detected in human cancers are indicated.

response[31-33], whereas Smad2 (Xmad2) induces dorsal mesoderm, an activin or Vg-1 response[32]. SMAD molecules are well conserved and act across species: human Smad1 (ref. 34), as well as *Drosophila* Mad[28], has ventralizing activity on *Xenopus* mesoderm, and mouse[35] and human[36] Smad2 have dorsalizing activity.

A picture is emerging in which different pathway-restricted SMADs couple to different receptors (Table 1). Smad2 and Smad3 are phosphorylated and translocated to the nucleus after stimulation by TGF-β[36-38] or activin (ref. 39 and A. Shimizu *et al.*, unpublished observation). Smad2 and Smad3 are very similar in their structures (Fig. 2a), and it is not surprising that there may be some redundancy in the functional activity between these two members of the family. Smad1 is phosphorylated and translocated into the nucleus after stimulation with BMP-2 (refs 29, 40) or BMP-4 (ref. 34). Smad5 induces ventral mesoderm in *Xenopus*[41], and the recently described Smad9/MADH6 (ref. 42) is structurally similar to Smad1 and Smad5; these molecules may also be involved in BMP signalling. There are reports that TGF-β also induces the phosphorylation of Smad1 (refs 43, 44), which may reflect redundancy or cooperativity in signalling (or a crossreactivity of antisera).

The phosphorylation of pathway-restricted SMADs by type I receptors triggers their activation. In the most C-terminal regions, pathway-restricted SMADs have a characteristic Ser-Ser-X-Ser (SSXS) motif, the two-most C-terminal serine residues of which are phosphorylated by type I receptors[40,45-47]. Pathway-restricted SMADs bind directly to type I receptors, as demonstrated by the co-immunoprecipitation of Smad2 or Smad3 with the type I and type II receptors affinity-crosslinked with ^{125}I-labelled TGF-β (refs 37, 38, 45). The association between TβR-I and Smad2 or Smad3 is dependent on the kinase activity of TβR-II, but was seen only with the kinase-inactive form of TβR-I and not with wild-type TβR-I (refs 38, 45). Moreover, the phosphopeptide maps of Smad1 phosphorylated in BMP-stimulated cells were similar to those of Smad1 phosphorylated *in vitro* by purified BMP type I receptor[11]. Taken together, these data suggest that the pathway-restricted SMADs are direct substrates of the type I receptor kinases, although a possible involvement of other kinases in SMAD activation has not been excluded. The data also suggest that the interaction between pathway-restricted SMADs and type I receptors is transient; presumably, SMADs are released from the receptors after phosphorylation. This idea is further supported by the observation that Smad2 molecules mutated at the three serine residues in the SSXS motif stably bind to the receptor and have dominant-negative effects[45-47].

Common-mediator SMADs. The mode of action of Smad4 differs from those of other members of the SMAD family. After ligand stimulation and phosphorylation of pathway-restricted SMADs, Smad4 forms hetero-oligomers with pathway-restricted SMADs[37,40,48,49], which in turn translocate into the nucleus and activate transcriptional responses (Fig. 3a). In mammalian cells, Smad4 forms complexes with Smad2 and Smad3 after activation of TGF-β or activin type I receptors[38,48,49], whereas it forms complexes with Smad1 (refs 40, 48), and possibly with Smad5 and Smad9, after activation of BMP type I receptors. Consequently, injection of Smad4 messenger mRNA into *Xenopus* animal caps induces both ventral and dorsal mesoderm[48,50] through the formation of complexes with Smad1, Smad5 or Smad9 and Smad2 or Smad3, respectively. Smad4, which lacks the C-terminal SSXS motif, does not bind to, nor is it phosphorylated by, TGF-β or BMP receptors[37,38,45,48]. The phosphorylation of Smad4 has been reported to increase after activin stimulation[18], although the functional importance of this remains to be determined.

So far, only Smad4 has been identified as a common-mediator SMAD in vertebrates. A Smad4 homologue has been identified in *Drosophila* (ref. 51; and P. Das and R. W. Padgett, personal com-

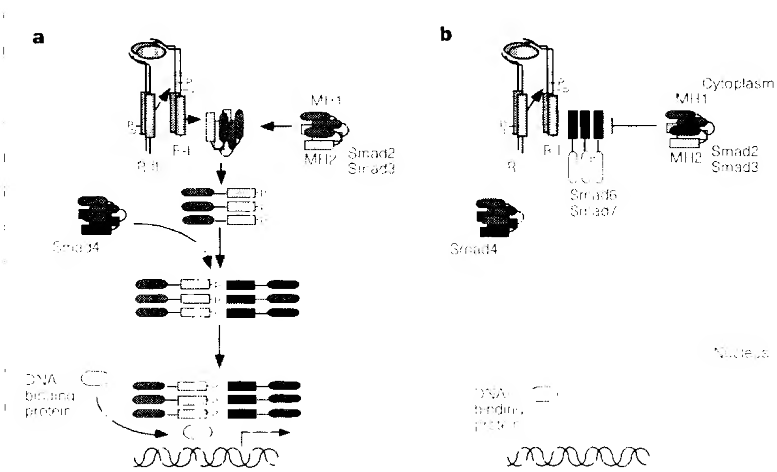

Figure 3 Agonistic and antagonistic SMAD proteins in TGF-β signalling. a, A hypothetical signal-transduction pathway for TGF-β. TGF-β binding leads to the assembly of a hetero-tetrameric receptor complex in which the type II receptor phosphorylates and activates the type I receptor. Pathway-restricted SMADs (Smad2 and Smad3) which may be anchored in the cytoplasm in homotrimeric forms, are phosphorylated, which leads to heteromerization with Smad4, a common-mediator SMAD. The hetero-oligomeric complex is then translocated to the nucleus, where it binds directly or in complex with other component(s) to DNA and affects transcription of specific genes. Note that it is not known if the hetero-oligomer between Smad2, Smad3 and Smad4 is a hexamer or has another stoichiometry. b, Inhibitory SMADs (Smad6 and Smad7) bind to the receptors, and prevent the phosphorylation and signalling activity of pathway-restricted SMADs. Whether inhibitory SMADs occur as monomers or multimers is not known.

munication) and *C. elegans* (Sma-4). The presence of homologue of both pathway-restricted SMADs and common-mediator SMADs in lower species suggests that complex formation of these two types of SMAD molecules may be a conserved mechanism for signalling downstream of serine/threonine kinase receptors.

Functional roles of SMAD/MAD domains. Structural studies and studies of SMAD mutants have now provided an insight into the functional roles of the different domains in SMADs (summarized in Fig. 4).

The MH2 domain of SMADs may serve as an effector domain in signal transduction, as suggested by its ability to induce a transcriptional response when fused to a yeast GAL4 DNA-binding domain[41] and by the finding that the MH2 domain of Smad2 induces a full range of activin responses in the absence of the MH1 domain[35]. However, in Smad4 the MH2 domain alone is not sufficient for signal transduction: a part of the linker region (termed the Smad4 activation domain[52]) that is not conserved in other SMADs is required for signalling activity, together with the MH2 domain[50,52]. Moreover, the MH2 domains of Smad1, Smad2 and Smad3 mediate homomeric interactions and are responsible for activation-induced interactions with Smad4 (refs 50, 53, 54).

In the resting cell, SMADs are localized in the cytoplasm. Stimulation with ligand leads to translocation to the nucleus. The observation that mouse Smad2 containing the linker region and MH2 domain localizes to the nucleus in the absence of ligand stimulation[35] suggests that the MH1 domain of Smad2 anchors the molecule in the cytoplasm. An alternative possibility is that truncation or ligand-induced heteromerization of SMADs leads to exposure of nuclear targeting sequence(s) in the linker region or the MH2 domain[50].

The MH1 domain of Smad4, and possibly of other SMADs, plays a role as a negative regulator by interacting with the MH2 domain, thereby preventing hetero-oligomer formation between pathway-restricted SMADs and common-mediator Smad[54]. Phosphorylation of the C-terminal SSXS motif in pathway-restricted SMADs appears to remove this inhibition. Mutations in SMAD MH1 domains have been reported in certain cancers, at Arg 133 in Smad2 and at the corresponding Arg 100 in Smad4 (refs 36, 55); these mutant SMADs form homo-oligomers but cannot form Smad2–Smad4 hetero-oligomers and cannot transduce signals. This inhibition occurs by increased affinity of the mutated MH1 domains to the corresponding MH2 domains, which leads to an augmentation of the auto-inhibitory function of the MH1 domain[54].

In addition to its role as a repressor of the MH2 domain, the MH1 domain, together with part of the linker region, may also be involved in direct DNA binding as indicated by studies on *Drosophila* Mad[56]. In *Drosophila*, Mad mediates the Dpp-dependent transcription of the *vestigial* (*vg*) gene through a sequence-specific DNA-binding activity. Binding of Mad to the 'quadrant' enhancer of *vg* was observed, but only when the MH2 domain was removed[56]. Thus, for the binding of the MH1 domain of Mad to the *vg* quadrant enhancer, the MH2 domain appears to have a repressor function. It remains to be determined whether mammalian SMAD MH1 domains also have direct DNA-binding activity.

Three-dimensional structure of SMADs. The three-dimensional structure of the MH2 domain of Smad4 has been determined by crystallography at 2.5 Å resolution[57]. It consists of a β-sandwich with antiparallel β-sheets, capped at one end by a three-α-helix bundle and at the other by three large loops and an α-helix (termed the loop/helix region). The MH2 domain of Smad4 forms a homotrimer in the crystal with the loop/helix region of one subunit interacting with the three-α-helix bundle of another. Size estimates by gel chromatography support the notion that the MH2 domain, as well as the intact Smad4 molecule, occur as trimeric structures in solution as well[57]. The MH2-domain trimer is in the form of a disc, with the amino terminals of all monomers, where the MH1 domains would be attached, phasing the same side. The other side of the disc may interact with other SMAD homotrimers in the heteromeric complex. The mutations in MH2 domains detected in human cancer cells may disrupt the structure of the oligomer: some mutations disrupt the folding of the protein; others, located at the loop/helix region or the three-α-helix bundle, prevent the formation of the homotrimer; others, located at a loop (L3) in the loop/helix region which is exposed on the surface of the disc, disrupt the formation of heteromers but not of homotrimers, indicating that this region may be exposed on the surface of the disc and be critical for heteromer formation[57]. In all cases, the assembly of heteromeric complex is prevented and the cell is deprived of antiproliferative TGF-β signals.

Activation of SMADs. Maximum transcriptional effect requires the cooperation between pathway-restricted SMADs and Smad4 (refs 37, 38, 48). Activation of type I receptors triggers the assembly of heteromeric complexes of the two types of SMADs, by phosphorylation of pathway-restricted SMADs in their C-terminal SSXS motifs. The mechanism may involve a phosphorylation-induced unfolding of the N- and C-terminal domains, allowing interaction with Smad4 to occur, and/or a direct interaction between the phosphorylated tail of pathway-restricted SMADs and Smad4 (ref. 46). Given the trimeric structure of Smad4 (ref. 57), such complexes may be hexamers, but their exact stoichiometry is unknown. Observations suggesting that other configurations of the active complex are possible is that full activity in a transcriptional assay can only be achieved when Smad2, Smad3 and Smad4 are all present, and that not only does Smad4 interact with Smad2 and Smad3, but Smad2 and Smad3 also interact with each other in a TGF-β-dependent manner[38].

Inhibitory SMADs. Smad6 and Smad7 diverge structurally from other members of the SMAD family[58–61]: whereas they share sequence similarity with other SMADs in their C-terminal domains, their N-terminal regions (36% identical between Smad6 and Smad7) differ from those of other SMADs. Inhibitory Smads have also been detected in *Xenopus* (Smad8; J. Christian, personal communication) and *Drosophila* (Dad; ref. 62). Smad6 and Smad7 function as inhibitors of TGF-β, activin and BMP signalling. They bind to type I receptors and interfere with the phosphorylation of the pathway-restricted SMADs. Consequently, active heteromeric Smad complexes are not formed. A requirement for binding of inhibitory SMADs to type I receptors is the activation of type I

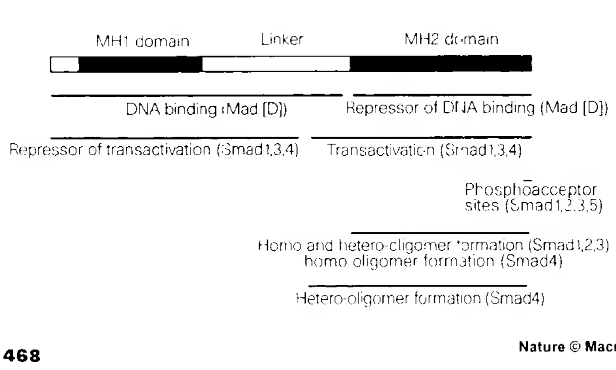

Figure 4 Different functional domains in SMADs. The conservation of MH1 and MH2 domains in the SMADs suggests that these domains may have similar functions in different members of the SMAD family, but it remains to be shown to what extent observations made on individual members (in parentheses) can be generalized.

receptor by type II receptor kinase. However, inhibitory SMADs show a more stable interaction with type I receptors than do pathway-restricted SMADs. As pathway-restricted SMADs can compete with inhibitory SMADs for binding, a plausible mechanism for inhibition is to prevent the receptor interaction and phosphorylation of pathway-restricted SMADs (Fig 3b). In an analogous way, Dad blocks the *Drosophila* phenotype induced by activated receptor or Mad[62], suggesting that Dad may directly interfere with the function of Mad.

Transcription of inhibitory SMAD mRNA is induced by stimulation by TGF-β as well as by other stimuli (ref. 59; and M. Kawabata *et al.*, unpublished observation), and in *Drosophila* Dad is induced by Dpp[62]. Thus, inhibitory SMADs may act as autoregulatory negative-feedback signals in the signal transduction of the TGF-β superfamily.

Transcriptional regulation by SMADs

TGF-β family members mediate their multifunctional effects by eliciting transcriptional responses on many target genes. The responsive elements in the promoters of some of these genes have been mapped and interacting transcription factors identified. In most cases, however, it is still unclear whether these genes are direct targets, and it is unknown whether SMADs are directly involved in their transcriptional regulation. Activin induces the transcription of the homeobox gene *goosecoid*[63] and the forkhead/winged-helix transcription factor *XFKHI* (ref. 64) without requirement of protein synthesis. Putative SMAD target genes in the BMP pathway include *Xom*[65], *Xvent*-1 (ref. 66) and *Msx*-1 (ref. 67). TGF-β potently induces transcription of plasminogen activator inhibitor-1 (ref. 68) and itself[69], with involvement of the AP-1 transcription factor, as well as the cyclin-dependent-kinase (CDK) inhibitors p15 (ref. 70) and p21 (ref. 71), with involvement of Sp1. Whether SMADs interact with AP-1 and Sp1 is not known.

Studies on the activation of *Xenopus Mix.2* and *Drosophila vg* provide the strongest evidence so far for a function of SMADs as transcriptional modulators downstream of serine/threonine kinase receptors. The homeobox gene *Mix.2* is an early-response gene induced by TGF-β superfamily members during early *Xenopus* development[72]. Smad2, Smad4 and FAST-1, a new member of the winged-helix transcription factor family, are components of an activin-responsive factor (ARF) that interacts directly in an activin-dependent manner with a 6-base-pair repeat in the activin-response element of the *Mix.2* promoter[72]. FAST-1 is the principal DNA-binding component in ARF. A C-terminal domain (amino acids 380–506) of FAST-1, termed SMAD-interacting domain (SID), is involved in binding to Smad2 and Smad4, and overexpression of SID specifically inhibits activin signalling[73]. The C-terminal part (amino acids 453–506) of SID is essential for the association of FAST-1 with Smad2, which occurs in the absence of Smad4. Phosphorylation of Smad2 enhances the interaction between Smad2 and FAST-1. The N-terminal part (amino acids 380–453) of SID appears to be required for the interaction of Smad4 to the Smad2–FAST-1 complex, but Smad4 does not directly bind FAST-1 in the absence of Smad2. Binding of Smad4 may stabilize the Smad2–FAST-1 complex as an active DNA-binding complex. The activin-response elements in promoters of *goosecoid* from different species[63], and *Xenopus XFKHI/XFD-1* (ref. 64) have been mapped and show little sequence similarity with the activin-response element in the *Mix.2* gene. This suggests that different SMAD-containing transcription factor complexes can be formed which show different DNA-binding specificities.

Drosophila Mad was shown to bind to a (G + C)-rich sequence and to be essential for activation of quadrant enhancer of *vg*[56]. The binding of Mad to DNA appears to be of low affinity, although it is specific, suggesting the need for heteromeric complex formation with a Smad4 homologue and/or other cofactors for high-affinity binding. This is also indicated by the finding that overexpression of the C-terminal domain of SMADs is sufficient to mimic the effects of ligand stimulation[35] and suggests that the principal DNA-binding component in the transcription factor complex is not provided by a SMAD. An intrinsic low-affinity DNA-binding activity in the MH1 domain may be complemented by specific interactions with other transcription factors through the MH2 domain.

Genetic analysis of Dpp signalling in *Drosophila* has implicated Schnurri (Shn) as an essential downstream component of Dpp-dependent signalling in embryonic endoderm pattern formation[74,75]. The *Shn* gene encodes a putative zinc-finger transcription factor with similarity to mammalian transcription factors of the major histocompatibility complex (MHC)-binding protein family. Shn activity, rather than its expression, appears to be regulated by Dpp. Thus, it is possible that Mad interacts directly with Shn to regulate transcriptional responses.

TGF-β family members may act as morphogens and induce concentration-dependent responses. These responses can be reproduced with increasing doses of SMADs[35,76], so it is possible that the level of nuclear SMAD provides a direct readout for the level of ligand-induced receptor activation. Promoters with different affinities for SMAD-containing transcription factor complexes may thus become activated in cells along a concentration gradient of TGF-β family members.

Receptor-interacting proteins

SMADs are clearly crucial for signal transduction of members of the TGF-β family. Using yeast two-hybrid screens, other molecules interacting with type I and type II serine/threonine kinase receptors have also been identified, which may modulate receptor signalling.

The FK506-binding immunophilin FKBP12 interacts with unstimulated TβR-I and other type I receptors. FKBP12 is not a substrate for the receptor kinase, and TβR-I mutants that are unable to interact with FKBP12 can still signal positively[77-79]. FKBP12 binds to a Leu-Pro sequence in the GS domain of type I receptors[77,79] (Fig. 1), and counteracts phosphorylation of the type I receptors by type II receptors; it is released from the type I receptors after ligand-induced receptor activation[78]. Thus, FKBP12 protects against ligand-independent, spontaneous activation of type I receptors by type II receptors[79].

Also, the α-subunit of farnesyltransferase can interact with TβR-I[80-82]. TGF-β stimulation does not alter the farnesyltransferase activity in mink lung cells, however, and this enzymatic activity is dispensable for the antiproliferative effect on TGF-β and its transcriptional responses in these cells[81]. Using a similar methodology, molecules that interact with TβR-II have also been identified—namely apolipoprotein J (ref. 83) and a WD-domain-containing protein, TRIP-1 (ref. 84), whose functional importance is not known.

Other cytoplasmic signalling pathways

In addition to the pathways already described, other parallel pathways may exist that could be important for the transduction of specific signals. Examples include TAK-1, a serine/threonine kinase of the MAP kinase kinase kinase family, which is activated by TGF-β or BMP-4 (ref. 85), and members of the Ras[86] or Rac[87] families of small GTP-binding proteins which also have been implicated in TGF-β signalling. Certain MAP kinases, such as the extracellular signal-regulated kinases (ERK)1 and 2 and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK), have also been reported to be activated by TGF-β in certain cell types[88,89]. Several of these pathways are efficiently activated in response to other signalling molecules. This may thus be yet another example of crosstalk between different signalling pathways, which appears to be common in signal transduction.

Subversion of signalling in tumorigenesis

TGF-β has a multifunctional role in tumorigenesis. At early stages,

when cells still respond to its antimitogenic effect, TGF-β may act as a tumour suppressor. However, during malignant progression, when cells acquire an insensitivity to growth inhibition by TGF-β, it may function as a tumour promoter by stimulation of angiogenesis, immunosuppression and synthesis of extracellular matrix, which provides an appropriate microenvironment for rapid tumour growth and metastasis. The biphasic action of TGF-β in tumorigenesis was demonstrated in a mouse skin model of multistage carcinogenesis using transgenic mice with keratinocyte-targeted TGF-β1 expression[90].

The escape from the antimitogenic response of cells by TGF-β during tumour progression suggests a potential function for components in the TGF-β signal transduction pathway as tumour suppressors[91]. Support for a tumour-suppressor role for the type II receptor of TGF-β came from the analysis of an inherited form of colon cancer with a microsatellite instability phenotype[92]. Moreover, the frequent mutation and homozygous deletion of *Smad4* in pancreatic cancers led to its original discovery as the *DPC4* tumour-suppressor gene[93] (Fig. 2b). Loss of Smad4 expression has been identified in various TGF-β-resistant cancer cells, and transfection of Smad4 in these cells rescues responsiveness to TGF-β[37,52,53]. The MH2 domain of Smad4 is often the target for point mutations and frameshift mutations that lead to premature stops. Mutations in the MH2 domain may disrupt the core structure of the protein, or perturb the ability to form stable homotrimers or hetero-oligomers with pathway-restricted SMADs, depending on which amino-acid residues are mutated[57] (see above). Somatic mutations in *Smad4* are frequently observed in pancreatic cancers[93], but less frequently in other types of cancers such as colon, breast and lung cancers. Functionally disruptive mutations in *Smad2*, a gene that is located close to Smad4 on chromosome 18, have so far been noted only in colorectal and lung cancers[36,94] (Fig. 2). *Smad1*, *Smad3*, *Smad5* and the MH2 domain of *Smad6* (*JV15-1*) do not appear to be frequently mutated in colon, breast, lung and pancreatic cancers[95]. The finding of a higher frequency of somatic mutations in *Smad4* than in other *Smad* genes is consistent with a unique and non-redundant role for the common partner Smad4 in TGF-β superfamily signalling.

TGF-β induces growth inhibition by upregulation of the CDK inhibitor p15 in certain epithelial cell lines[96]. However, analysis of p15-defective human cancer cell lines reveals that the antiproliferative effect of TGF-β is also mediated by repression of the expression of Cdc25A, a CDK tyrosine phosphatase which activates CDK[97]. Cdc25A is transcriptionally induced by c-Myc[98], and *c-myc* expression is repressed by TGF-β with similar kinetics to Cdc25A (refs 97, 99); an interesting possibility is therefore that c-Myc is involved in the transcriptional regulation of Cdc25A by TGF-β.

Conclusions

In view of their crucial importance in embryonal development, it is no surprise that signalling by TGF-β members is carefully regulated. Several intracellular control mechanisms have been discussed here. In addition, there are observations indicating that signalling is also regulated at several extracellular steps, including activation from latent precursor complexes, and interactions with specific binding proteins and accessory receptors.

Remarkable progress has been made in unravelling a signalling pathway for TGF-β and related molecules from the cell membrane all the way to the nucleus (Fig. 3). SMADs are key components in these signal transduction pathways. At present, nine vertebrate SMADs are known (Fig. 2a), but the family probably contains several other members. After phosphorylation and activation by receptor kinases, hetero-oligomeric SMAD complexes migrate into the nucleus and, either directly or in complex with other proteins, affect transcription of specific genes. Thus, the overall mechanism is reminiscent of that of signal transducers and activators of transcription (STAT) molecules, which, after phosphorylation by cytokine-receptor-associated JAK tyrosine kinases or tyrosine kinase receptors, dimerize and move into the nucleus and induce the transcription of specific genes[100].

An important finding is that certain SMADs serve as inhibitors in the signal transduction of members of the TGF-β superfamily by preventing the interaction between the serine/threonine kinase receptors and pathway-restricted SMADs. As expression of the inhibitory SMADs is induced by ligand stimulation, they may have a negative-feedback role in signal transduction. Likewise, feedback switch-off signals have been found in several other signal transduction pathways and emerge as a common theme in signal transduction.

Although we are gaining our first insight into the three-dimensional structure of SMAD[57] and the involvement of SMADs in direct[56] and indirect[73] binding to promoter regions in specific genes, many important issues remain unresolved. Is the basis for specificity of interaction between type I receptors and different SMADs due to specific docking epitopes or to the substrate specificities of the receptor kinases? Is receptor-induced phosphorylation in the C-terminal tail sufficient for SMAD activation and initiation of all subsequent downstream signalling events, or are other phosphorylation events necessary? By which mechanism do SMADs translocate from the cytoplasm to the nucleus? Which are the partners of SMADs in the transcriptionally active complexes, and which are the target genes? Given the level of activity in the field, answers are likely to come along soon.

Note added in proof: Tyrosine kinase receptor-mediated activation of MAP kinase was recently shown to lead to phosphorylation of Smad1 in the linker region and inhibition of its translocation to the nucleus[101]. Cross-talk between different types of signalling pathways may thus occur by differential regulation of Smad1 activation. □

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Exhibit A

Stem cell differentiation requires a paracrine pathway in the heart

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ABSTRACT **Members of the transforming growth factor β1 (TGF-β) superfamily—namely, TGF-β and BMP2—applied to undifferentiated murine embryonic stem cells up-regulated mRNA of mesodermal (Brachyury) and cardiac specific transcription factors (Nkx2.5, MEF2C). Embryoid bodies generated from stem cells primed with these growth factors demonstrated an increased potential for cardiac differentiation with a significant increase in beating areas and enhanced myofibrillogenesis. In an environment of postmitotic cardiomyocytes, stem cells engineered to express a fluorescent protein under the control of a cardiac promoter differentiated into fluorescent ventricular myocytes beating in synchrony with host cells, a process significantly enhanced by TGF-β or BMP2. In vitro, disruption of the TGF-β/BMP signaling pathways by latency-associated peptide and/or noggin prevented differentiation of stem cells. In fact, only host cells that secrete a TGF-β family member induced a cardiac phenotype in stem cells. In vivo, transplantation of stem cells into heart also resulted in cardiac differentiation provided that TGF-β/BMP2 signaling was intact. In infarcted myocardium, grafted stem cells differentiated into functional cardiomyocytes integrated with surrounding tissue, improving contractile performance. Thus, embryonic stem cells are directed to differentiate into cardiomyocytes by signaling mediated through TGF-β/BMP2, a cardiac paracrine pathway required for therapeutic benefit of stem cell transplantation in diseased heart.—Behfar, A., Zingman, L. V., Hodgson, D. M., Rauzier, J.-M., Kane, G. C., Terzic, A., Pucéat, M. Stem cell differentiation requires a paracrine pathway in the heart.** ***FASEB J.*** **16, 1558–1566 (2002)**

Key Words: TGF-β · ischemic heart disease · cardiomyocytes · cardioblasts

ISCHEMIC HEART DISEASE is a leading cause of heart failure precipitated by the death of highly vulnerable cardiomyocytes (1). The heart under stress primarily responds with cell hypertrophy rather than proliferation due to a limited mitotic capacity of differentiated cardiomyocytes (2). This restricts repair of the injured myocardium to replacement by fibrotic tissue disrupting proper contractile function despite the potential of some myocytes to participate in postinfarction regeneration (3). In this regard, a novel therapeutic approach has emerged based on repopulating injured tissue with cells of myogenic phenotype. Indeed, different cell types have been grafted into the heart, including myoblasts, cardioblasts, and fetal or neonatal cardiomyocytes (4–9). Although promising, use of already differentiated muscle cells suffers from several limitations. The cardiac area colonized by implanted cells is small due to a low dividing capacity of such terminally differentiated cells (3). Moreover, long-term survival and electrical coupling of these cells within the myocardium remains controversial (10, 11).

Recently, the plasticity of uncommitted stem cells has opened new perspectives in tissue regeneration (12–14). Adult bone marrow and hematopoietic stem cells have been successfully engrafted into ischemic hearts, differentiating into smooth muscle, endothelial, and cardiac phenotypes (15, 16). Evidence has been provided that such engrafted stem cells may improve the function of diseased heart (15). Although use of undifferentiated cells may resolve some of the limitations observed with differentiated myoblasts or myocytes, recent findings have called for caution regarding the use of adult stem cells that by fusion take the phenotype of recipient cells, negating a therapeutic benefit (17, 18). Indeed, much remains to be understood before stem cell-based therapies can be used effectively for cardiac repair.

One of the fundamental questions is how pluripotent stem cells respond to the host environment and differentiate toward a specific cell phenotype. More specifically, the molecular signals that induce commitment, proliferation, and differentiation of stem cells into cardiomyocytes within an infarcted myocardium are unknown (15).

Therefore, we focused our investigation on mecha-

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nisms underlying differentiation of stem cells grafted onto cardiomyocytes, normal, and postinfarcted myocardium. We report that members of the transforming growth factor β1 (TGF-β) superfamily commit undifferentiated stem cells into the cardiac lineage. The paracrine function of host cardiomyocytes in secreting TGF-β and/or BMP2 underlies cardiac differentiation of engrafted pluripotent stem cells, resulting in a gain-of-function of the infarcted myocardium. These findings favor the use of growth factor-committed stem cells for cell therapy in the diseased heart.

MATERIALS AND METHODS

Embryonic stem cell differentiation

The CGR8 murine embryonic stem cell line (19) was propagated in BHK21 medium supplemented with pyruvate, nonessential amino acids, mercaptoethanol, 7.5% fetal calf serum (FCS, Biomedia, Paris, France), and the leukemia inhibitory factor (LIF) obtained from LIF-D cells (20). Differentiation was carried out in hanging drops (21) of differentiation medium (BHK21 with 20% FCS without LIF) in which embryoid bodies were formed at 2 days (D0–2). Embryoid bodies were incubated for 3 days (D2–5) in suspension and for at least 7 days (D6–12) on gelatin-coated dishes or laminin-coated glass coverslips (20).

Generation of stem cell clones expressing a reporter gene

The CGR8 cell clone was engineered to express the enhanced cyan fluorescent protein (ECFP) under the control of the cardiac-specific α-actin promoter, subcloned upstream of ECFP using *Xho*I and *Hin*dIII restriction sites of the promoterless pECFP vector (Clontech, Palo Alto, CA). This α-actin promoter (actinECFP) construct was linearized using *Xho*I, electroporated into CGR8 stem cells, and colonies were screened for the construct by PCR after G418 treatment. Alternatively, the promoter of the ventricular myosin light chain 2 (MLC2v) was used to confirm ventricular differentiation (20). Some actinECFP stem cell clones were engineered to be resistant to TGF-β superfamily signaling through expression of noggin or a dominant negative mutant of the TGFβRII receptor (ΔKTGFβRII) (22). Noggin or ΔKTGFβRII cDNA were subcloned in the pcDNAhygromycin vector (Clontech) using *Xho*I and *Bam*HI or *Kpn*I and *Hin*dIII restriction sites, respectively. After electroporation of linearized plasmids into CGR8 stem cells, hygromycin-resistant colonies were screened by PCR for construct expression.

RT-PCR and real-time quantitative PCR

Total RNA was prepared from CGR8 stem cells as described (20, 23). After reverse transcription, 10 ng cDNA was used for real-time quantitative PCR, performed with a Lightcycler and the SYBR green fast start kit (Roche, Germany). Primers used in real-time PCR were as follows: Brachyury forward 5′-GACTTCGTGACGGCTGACAA-3′ and reverse 5′-CGAGTCTGGGTGGATGTAG-3′; MEF2C forward 5′-AGATACCCACAACACACCACGCGCC-3′ and reverse 5′-ATCCTTCAGAGAGTCGCATGCGCTT-3′; Nkx2.5 forward 5′-CATTTTACCCGGGAGCCTACGGTG-3′ and reverse 5′-GCTTTCCGTCGCCGCCGTG-3′; β-tubulin forward 5′-CCGGACAGTGTGGCAACCAGATCGG-3′ and reverse 5′-TGGCCAAAAGGACCTGAGCGAACGG-3′; Myf 5 forward 5′-GGAGATCCTCAGGAATGCCAT-3′ and reverse 5′-TGCTGTTCTTTCGGGACCAGA-3′; ICAM forward 5′-GACTTCACCATTGAGTGCACGGTGT-3′ and reverse 5′-TATACCCACCATGGGGCCGTAGATC-3′. The reaction contained 1 μL of Master SYBR green I mix (*Taq* DNA polymerase, buffer, deoxynucleoside trisphosphate mix, and SYBR green I dye), 3 mM $MgCl_2$, and 0.5 μM of each primer to which 2 μL of diluted cDNA was added. A standard concentration curve was established by serial dilution of gel-purified gene specific PCR fragments. Data were normalized using β-tubulin as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95°C for 8 min, 45 cycles of denaturation at 95°C for 3 s, annealing at 60–65°C for 8–10 s, and extension at 72°C for 7–10 s performed at a temperature transition rate of 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve acquired by heating the product to 95°C, cooling to and maintaining at 70°C for 20 s, then slowly (0.3°C/s) heating to 95°C was used to determine the specificity of PCR products, confirmed by gel electrophoresis.

Cell imaging

Fluorescent images of EBs or isolated cells were acquired on a LEICA microscope with objectives mounted on a piezoelectric device, digitized on-line with a Micromax 1300YHS CCD camera (Princeton, NJ), and stored as volume files ('stack' of z-section images) using the Metamorph software (Universal Imaging, Downington, PA). ECFP was detected in embryoid bodies or isolated cells illuminated with a Hg lamp at 400 ± 20 nm with CFP fluorescence recorded by a X114–2 CFP LEICA filter cube that consists of a DM 455 dichroic mirror and a 480 ± 30 nm emission filter. Beating areas in embryoid bodies were identified by videomicroscopy using the stream acquisition mode and their size quantified with the region measurement option of Metamorph. The sum of individual beating areas was normalized to the total size of the embryoid body. Embryoid bodies (12-day-old) were fixed in 3% paraformaldehyde for 30 min, permeabilized for 30 min with 1% Triton X-100, and immunostained as described (20). In situ immunostained sarcomere-specific proteins, actinin, or MLC2v were visualized in 0.1 μm optically z-sectioned embryoid bodies. Similarly, immunohistochemistry of heart sections was performed on paraffin sections. To improve resolution and signal-to-noise ratio, images were restored using Huygens software (Huygens 2.2.1, Scientific Volume Imaging, Hilversum, The Netherlands) and visualized using Imaris (Bitplane, Switzerland). Calculations were performed on an Octane workstation (Silicon Graphics, Los Angeles, CA).

Coincubation of stem cells and cardiomyocytes

Cardiomyocytes isolated from 2- to 3-day-old neonatal rat ventricles and purified on a Percoll (Pharmacia, Uppsala, Sweden) gradient (24) were plated at a density of 2×10^5 cell per 35 mm dishes and cultured for 3 days. At this stage, cells are postmitotic and do not dedifferentiate in culture (25). Coincubation was then performed with 10^4 CGR8 stem cells expressing ECFP under the control of either the cardiac-specific α-actin or ventricular specific MLC2v promoter, untreated or treated with the 2.5 ng/mL TGF-β or 5 ng/mL bone morphogenetic protein (BMP2) (R&D System, Abingdon, UK), resuspended in differentiation medium, and added to the cardiomyocyte-containing dish. Cardiac differentiation of stem cells was monitored by appearance of ECFP fluorescence using videomicroscopy (20).

Coculture of stem cells with BMP-secreting or nonsecreting C3H10T1/2 cells

Native C3H10T1/2 cells were cultured in DMEM supplemented with 10% FCS. The C9 cell clone, secreting BMP2, was engineered from the C3H10T1/2 cell line using an inducible BMP2 expression vector (ptTATop-BMP2). C9 cells were routinely cultured in the presence of 1 μg/mL doxycycline to prevent premature BMP2 expression (26). Next, 10^4 CGR8 stem cells expressing ECFP under the control of the cardiac α-actin promoter were cocultured with confluent native C3H10T1/2 or BMP-secreting C9 cells in the absence of doxycycline.

In vivo stem cell injection

CGR8 stem cells (5×10^5) expressing ECFP under the control of the actinECFP with or without noggin or the ΔKTGFβRII mutant (22) were trypsinized, resuspended in serum free medium, and injected in isoflurane-anesthetized (3% induction; 1.5% maintenance) mice. Injections were made through the left chest wall as well as via an abdominal approach through the diaphragm into the left ventricle using a 26 gauge needle. Three to 4 wk later, hearts were excised, cryofixed, and sliced in 4 μm-thick sections at 0.5 mm increments through the tissue. ECFP expression was visualized using a Zeiss Axioplan epifluorescence wide field microscope.

Myocardial infarction model

Myocardial infarction was induced in both Sprague-Dawley and Wistar rats by in situ ligature of the left coronary artery (27). Four weeks after surgery, rats were anesthetized with ketamine (70 mg/kg) and xylazine (15 mg/kg) or isoflurane (3% induction; 1.5% maintenance) and the heart was exposed after thoracotomy. Concomitantly, trypsinized $3 \cdot 10^5$ cells (suspended in 20 μL medium) or medium without cells (sham) injected along the border zone of the infarcted area at three different locations (below the left atrium, in the middle portion of the left ventricle, and at the apex) using a 27 gauge needle. Five weeks later, animals were either killed with phenobarbital or assessed for function with echocardiography. Hearts from killed animals were rapidly removed; ventricles were sliced in two transverse sections fixed with 4% paraformaldehyde. The infarcted area with surrounding tissue was embedded in paraffin and successive 5 μm sections were cut for immunohistology. Sections were stained with an anti-MLC2v antiserum (20), anti-connexin 43 polyclonal antibody (Sigma France) or the Verhaeff-Van Gieson stain for collagen and elastin (IMEB Inc., San Marcos, CA). Echocardiography was performed on isoflurane-anesthetized rats using a 5 MHz transducer on an ultrasonographic scanner (Vingmed System FiVe, GE Medical Systems, Milwaukee, WI). Parasternal short axis views with M-mode were acquired at the ventricular base immediately distal to the mitral valve. Ejection fraction was calculated as follows: $EF = (S^2 - D^2)/D^2 \cdot 100$, where S is the systolic and D the diastolic dimension (expressed in cm).

RESULTS

TGF-β and BMP2 up-regulate cardiac transcription factors in embryonic stem cells

To determine whether undifferentiated stem cells could be specifically committed to a cardiac cell lineage by growth factors, embryonic stem cells were treated (24 h) with TGF-β or BMP2 in 3.5% or 7.5% FCS-containing medium in the presence of LIF. Stem cells deprived of LIF, a suppressor of differentiation, lose their compact appearance and with it the potential for mesodermal differentiation (**Fig. 1**). Real-time quantitative PCR revealed that TGF-β and BMP2 both significantly up-regulated mRNA levels of Brachyury, a mesodermal transcription factor (Fig. 1*a*). TGF-β and BMP2 also significantly increased mRNA encoding Nkx2.5 (Fig. 1*b*) and MEF2C (Fig. 1*c*), early and late markers in cardiac differentiation (28), while maintaining colony morphology (Fig. 1*d*). In preliminary experiments, concentration response curve revealed that induction of transcription factors was most effective at 2.5 and 5 ng/mL of TGF-β and BMP2, respectively. In contrast, expression of Myf5, a skeletal muscle marker, or ICAM, an endothelial cell marker, was not significantly up-regulated (data not shown). Thus, TGF-β growth factor family members promote in stem cells induction of the cardiac gene program.

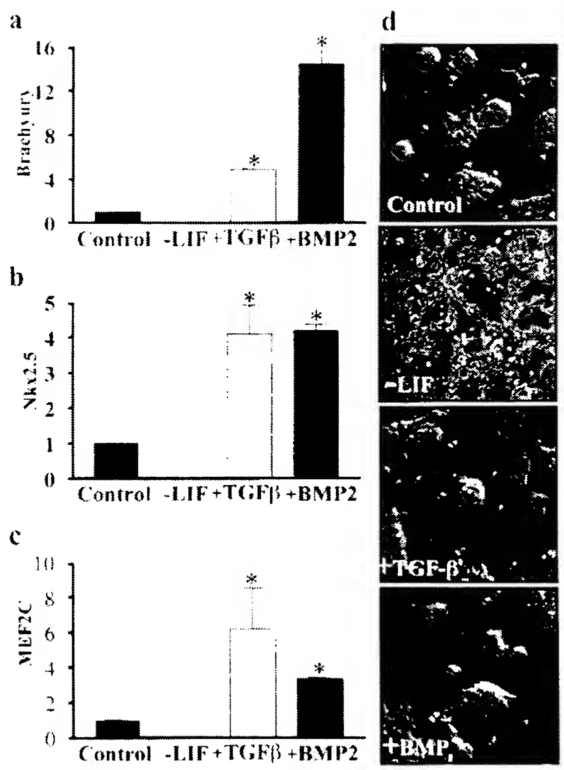

Figure 1. TGF-β- and BMP2-induced cardiac commitment of embryonic stem cells. CGR8 stem cells were left untreated (control) or were treated for 24 h with TGF-β (2.5 ng/mL) or BMP2 (5 ng/mL) in a low-serum and LIF-containing medium. Alternatively, cells were grown in media without LIF, TGF-β, and BMP2 (−LIF). Expression of mRNA for Brachyury (*a*), Nkx2.5 (*b*), and MEF2C (*c*) was measured using real-time quantitative PCR. Data are means (±SE; $n=3$ each) of the ratio between expression of gene of interest vs. tubulin presented as multiples of control. *Significant difference from control ($P<0.01$). *d*) Morphology of stem cell colonies under different experimental conditions.

TGF-β and BMP2 induced-commitment of ES cells favors cardiac differentiation within embryoid bodies

Embryonic stem cells unprimed or primed with TGF-β and BMP2 (24 h) were allowed to differentiate within embryoid bodies (20, 21). Within 7 days, beating clusters appeared within the mesodermal layer (**Fig. 2*a***). On day 9, the time required for maximal beating activity (20), contracting areas were threefold larger in embryoid bodies formed from growth factor-primed stem cells compared to untreated controls (Fig. 2*b*). Immunostaining of actinin, a protein distributed in z-discs of sarcomeres, revealed extensive myofibrillar regions in embryoid bodies from stem cells treated with TGF-β or BMP2, in contrast to smaller areas in controls (Fig. 2*c–e*, left panels). Enhanced myofibrillogenesis induced by TGF-β or BMP2 was not detrimental to sarcomeric architecture, which remained highly organized (Fig. 2*c–e*, right panels). Accordingly, similar beating rates (beats/s) of 1.2 ± 0.3 (n=5), 1.1 ± 0.3 (n=5), and 1.0 ± 0.2 (n=10) and sarcomeric unit sizes (in μm) of 2.0 ± 0.2 (n=10), 1.9 ± 0.3 (n=5), and 1.8 ± 0.3 (n=5) were observed in embryoid bodies from controls, TGF-β- and BMP2-primed stem cells, respectively. Thus, pretreatment of embryonic stem cells with TGF-β growth factor members results in embryoid bodies with greater areas of cardiac differentiation and normal sarcomeric organization.

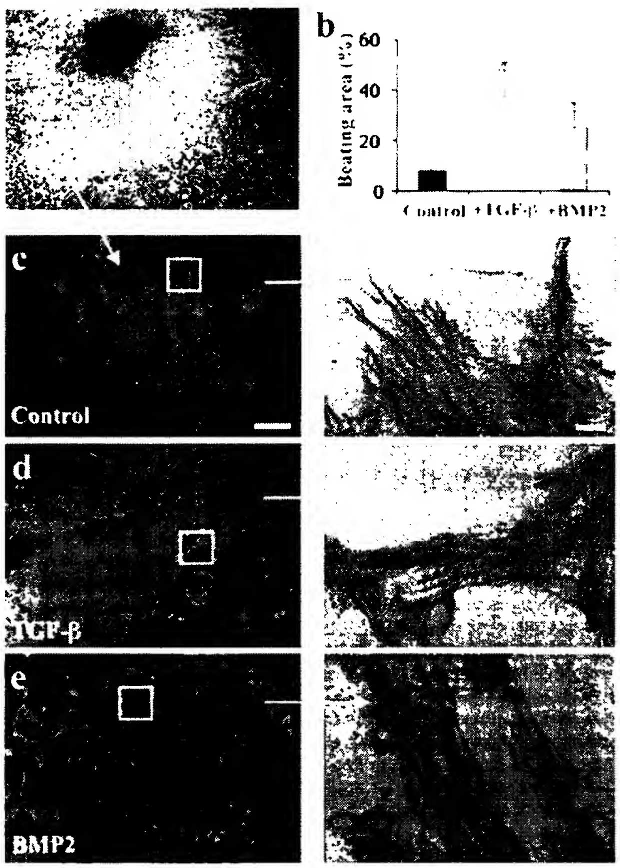

Figure 2. TGF-β and BMP2 enhance the potential of stem cells for cardiac differentiation in embryoid bodies. Embryoid bodies were formed in a hanging drop from untreated CGR8 stem cells (*a*) or stem cells primed with TGF-β and BMP2. On day 7 after initiation of differentiation, beating clusters were observed within the mesodermal layer. *b*) Total beating area measured on day 9 and expressed as percentage of total embryoid body area. Data were obtained from at least 3 experiments in each group. Embryoid bodies generated from untreated stem cells (*c*) or cells treated for 24 h with TGF-β (*d*) or BMP2 (*e*) were fixed and immunostained with an anti-actinin antibody and a secondary FITC-conjugated antibody. *c–e*) Left panels: 10× magnification, yellow bar indicates 100 μm. Right panels: 63× magnification; yellow bar indicates 15 μm.

In vitro cardiac differentiation of stem cells grafted onto cardiomyocytes enhanced by TGF-β and BMP2

Undifferentiated stem cells carrying the fluorescent ECFP probe under the control of the cardiac α-actin promoter (actinECFP cells) failed to emit fluorescence in the absence of a host cellular milieu (not illustrated). However, when grafted onto postmitotic isolated ventricular cardiomyocytes, actinECFP stem cells expressed ECFP vigorously after 5 days of coculture (**Fig. 3*a***), indicating that the cardiac α-actin promoter was turned on. In fact, cells expressing ECFP fluorescence demonstrated immunostaining of the ventricular marker myosin light chain 2 (MLC2v; Fig. 3*a*, inset), indicating ventricular phenotype. Similarly, stem cells expressing ECFP under the control of the MLC2v promoter (20) instead of the α-actin promoter, also fluoresced within 7 days in the presence of host cardiomyocytes (data not illustrated). Cardiac differentiation of stem cells was augmented by pretreatment with TGF-β (5 ng/mL) or BMP2 (2.5 ng/mL) before engraftment onto host cardiomyocytes (Fig. 3*b–d*). All differentiated stem cells displayed positive staining for connexin 43 (Fig. 3*e*), a gap junction protein required for intercellular communication expressed in ventricular myocytes (29) and ES cell-derived cardiomyocytes (30). Indeed, the beating pattern of stem cell-derived cardiomyocytes and host cardiac cells was synchronous on time-lapse videomicroscopy (data not shown). Thus, cardiac cells provide an environment in which stem cells can differentiate into cardiac lineage, an effect enhanced by stem cell priming with TGF-β growth factor members.

Differentiation of stem cells induced by TGF-β/BMP2 paracrine signals from host cells

The heart harbors an endocrine function (31). Cardiomyocytes and cardiac fibroblasts release members of the TGF-β family, including TGF-β (32, 33) and BMP2 (34), involved in embryonic cardiomyogenesis (35). Cardiac differentiation of actinECFP stem cells in coculture with postmitotic ventricular cardiomyocytes (**Fig. 4*a***) was disrupted by the blockers of TGF-β or BMP2 receptor-mediated signaling (36, 37). Latency-associated peptide (LAP; Fig. 4*b*) and noggin (Fig. 4*c*) used individually or in combination (Fig. 4*d*) prevented

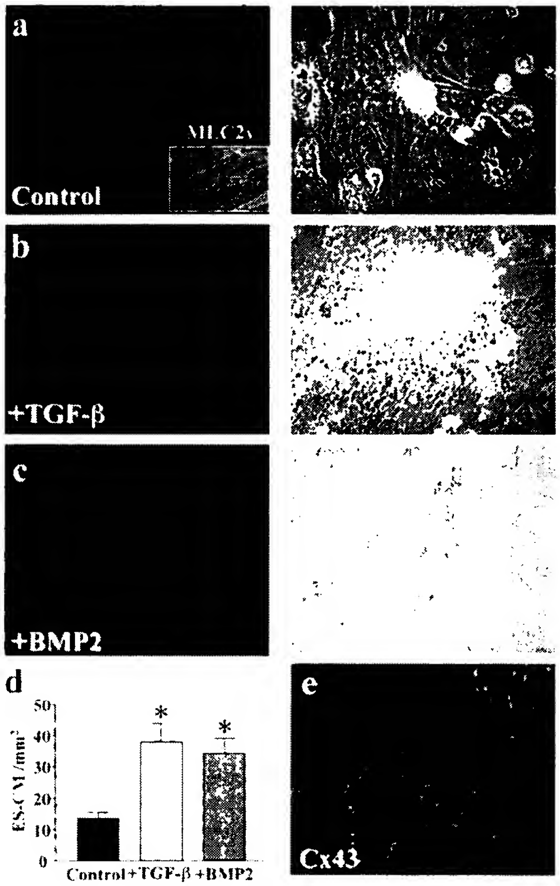

Figure 3. TGF-β and BMP2 commit stem cells grafted onto cardiomyocytes in vitro. Stem cells expressing ECFP under the control of the α-actin promoter were cocultured with ventricular cardiomyocytes. *a)* On day 5 after plating stem cells, cyan fluorescence was observed as was red MLC2v immunostaining (inset). *b–d)* Stem cells pretreated for 24 h with TGF-β (*b*) and BMP2 (*c*) displayed enhanced cyan fluorescence corresponding to a greater number of fluorescing embryonic stem cell-derived cardiomyocytes (ES-CM; *d*). *Significant difference from control ($P<0.01$, $n=3$ in each group). *a–c)* Transmitted light microscopy images are shown to the right of corresponding fluorescent images. *e)* Connexin 43 (Cx43) antibody was used to detect integration of differentiated stem cells with host cardiomyocytes. The panel is typical of multiple images obtained from 3 separate cocultures.

cardiac differentiation of stem cells as shown by the absence of ECFP expression. In the presence of LAP and noggin, stem cells formed clusters of undifferentiated cells (Fig. 4*b–d*), and the number of stem cell-derived cardiomyocytes was essentially negligible compared to control (Fig. 4*e*). Removal of LAP and noggin while in coculture with cardiomyocytes allowed stem cells to regain the ability to differentiate and express ECFP (Fig. 4*f*).

To directly assess the role of a host cell and of cell–cell interactions in stem cell differentiation, actin-ECFP cells were cocultured with embryonic C3H10T1/2 fibroblasts, wild-type, or genetically engineered as the C9 cell clone to secrete BMP2. Cardiac differentiation of actinECFP stem cells manifested through expression of fluorescence, was detected within 5–7 days in cells cocultured with the BMP2-secreting C9 cell clone, but not with C3H10T1/2

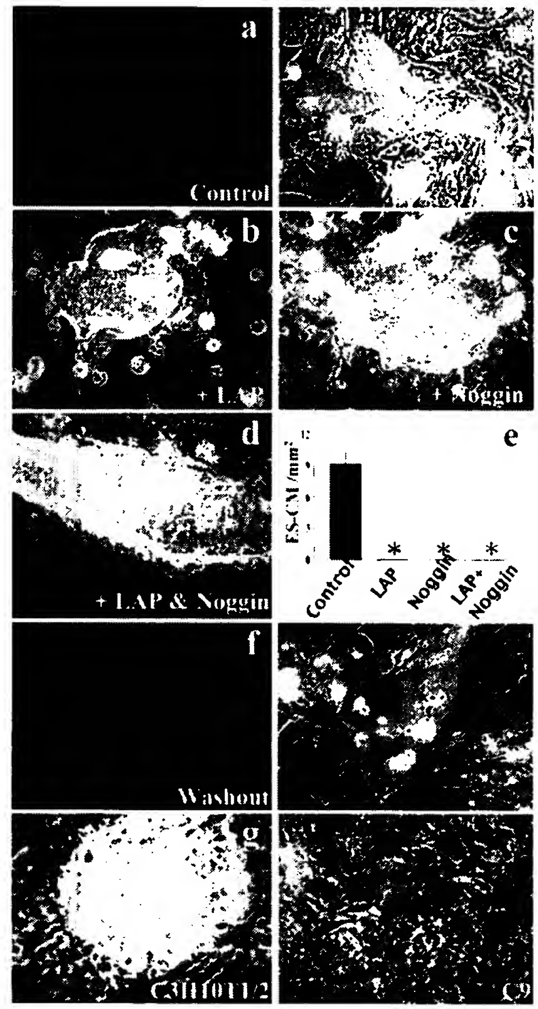

Figure 4. TGF-β BMP2 paracrine signals from host cells differentiate stem cells. *a–f)* Stem cells expressing ECFP under the control of the α-actin promoter were cocultured with cardiomyocytes for 5 days. *a)* ECFP fluorescence (left) and phase (right) microscopy reveal cardiac differentiation of stem cells. Five day treatment of cocultures with latency-associated peptide (LAP, *b*), noggin (*c*), or both (*d*) prevented expression of cyan fluorescence indicating negligible cardiac differentiation. The concentration of LAP (5 nM) and the volume of the conditioned medium from CHO-expressing noggin were chosen for their efficacy to inhibit cardiac differentiation of stem cells within embryoid bodies. *e)* Data from 3 cocultures in each group expressed as mean (±SE) of the number of ECFP-expressing cells (ES-CM)/mm² of the microscope field. *f)* Within 2 days of removal of LAP and noggin, stem cells differentiated into ECFP-expressing cardiomyocytes. *g)* Whereas wild-type C3H10T1/2 embryonic fibroblasts not secreting BMP2 did not promote stem cell differentiation to fluorescing cardiomyocytes in coculture (left), the C9 cell clone secreting BMP2 promoted cyan fluorescence expression within 7 days, indicating differentiation of stem cells into cardiomyocytes (right). The experiment was performed on 3 cocultures with similar results.

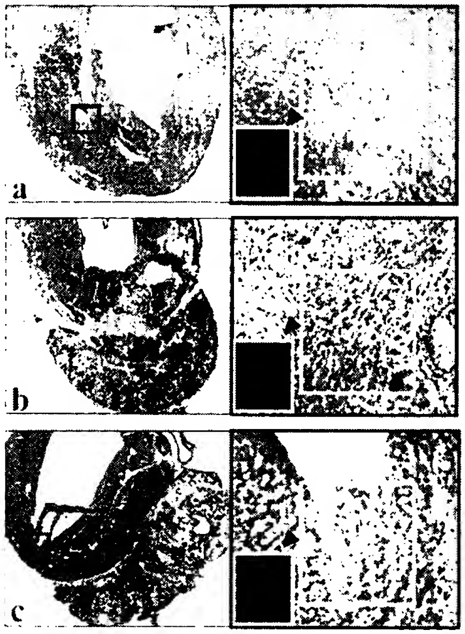

Figure 5. Paracrine stimulation by TGF-β family members required for in vivo stem cell differentiation. Hematoxylin-eosin stained cryosections of mouse hearts 4 wk after transplantation with stem cells. *a)* Heart injected with stem cells expressing ECFP under the control of the α-actin promoter (left) reveal fluorescent cardiomyocytes (right, inset) indicative of differentiated stem cells integrated within the host myocardium (right). *b, c)* Hearts injected with ECFP stem cells engineered to express noggin (*b*) or ΔKTGFβRII (*c*) reveal undifferentiated tumor formation with invasion of the myocardial wall (left) and absence of fluorescence (right, inset), indicating stem cells with compromised cardiac differentiation disseminated through the host myocardium (right). Right images are a magnification of the black inset on left images. Fluorescence was visualized in the area delimited by the yellow frame on the right images as indicated by arrows.

fibroblasts that do not secrete BMP2 (Fig. 4g). Thus, host cells through paracrine TGF-β/BMP2 signaling induce cardiac differentiation of stem cells.

In vivo stem cell differentiation requires paracrine stimulation by TGF-β family members

To assess stem cell differentiation in vivo, actinECFP cells were grafted into hearts of mice ($n=3$). After 4 wk, fluorescent myocytes expressing ECFP, indicative of stem cell differentiation, were integrated throughout the host myocardium (**Fig. 5*a***). In contrast, actinECFP cells engineered to express noggin or ΔKTGFβRII, disrupters of TGF-β family receptor-mediated signaling, failed to express ECFP fluorescence 4 wk post-transplantation, indicating the absence of cardiac differentiation ($n=6$, Fig. 5*b, c*). In fact, such stem cells incapable of responding to TGF-β receptor signaling remained undifferentiated and developed into invasive tumors in the hearts in one-third of the injected mice (Fig. 5*b, c*), an effect never seen from TGF-β-responsive stem cells ($n=20$, Fig. 5*a*). Thus, the paracrine effect of TGF-β family signaling is mandatory for proper cardiac differentiation of stem cells in vivo.

In vivo differentiation of embryonic stem cells in diseased myocardium improves contractile function

To test whether stem cells respond to TGF-β/BMP in vivo in a diseased condition, we used an established model of heart failure after myocardial infarction (27) in which cardiomyocytes are known to maintain secretion of TGFβ (38, 39). Pluripotent actinECFP cells were injected into the area surrounding the infarcted tissue (**Fig. 6*a, b***). After 5 wk, cells expressed ECFP fluorescence at the site of injection (Fig. 6*c*) as well as within the neovascularized scar tissue (Fig. 6*d–e*). Fluorescent cells displayed a typical cardiac phenotype, including sarcomeric striations (Fig. 6*f*), and immunoreactivity for MLC2v, a ventricle-specific myosin light chain isoform (Fig. 6*g*). Immunostaining for the gap junction

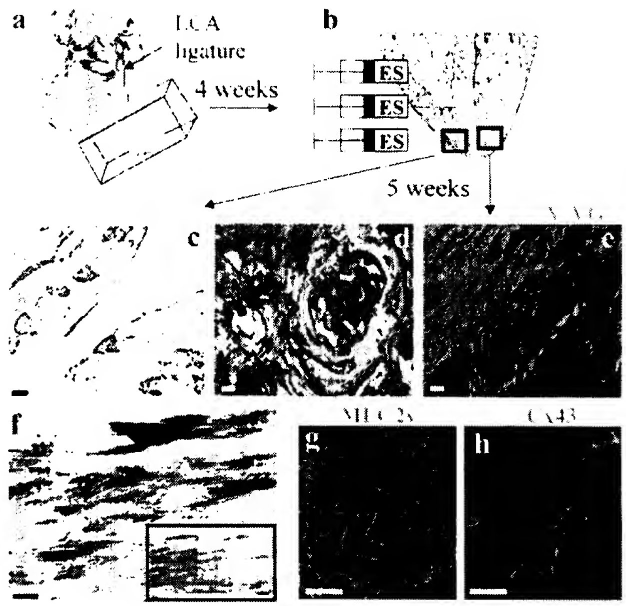

Figure 6. In vivo differentiation of stem cells in infarcted myocardium. *a)* Ligature of the left coronary artery (LCA) in rat causes myocardial infarction with development of cardiac failure within 4 wk. *b)* Stem cells expressing ECFP under the control of the cardiac α-actin promoter were injected into three peri-infarct locations 4 wk after infarction. *c–e)* Paraffin-embedded heart sections (5 μm) were imaged 5 wk after stem cell transplantation. Digital restoration of a z-stack of images acquired from the apical site of injection (*c*) or within the scar tissue (*d, e*) demonstrates numerous ECFP-expressing cells. *d)* Note the presence of neovascularization. *d, e)* Sections were stained with Verhaeff-Van Gieson (V-VG) to highlight collagen (in red) and elastin (black fibers around an erythrocyte-containing vessel). *f)* A digitally restored z-stack of images acquired at high magnification further reveals that stem cell-derived ECFP-expressing cardiomyocytes generated a network of myofibrils. Inset: magnified sarcomeres. *g, h)* Immunostaining of MLC2v (*g*) and connexin 43 (Cx43; *h*) in ECFP-expressing stem cell-derived cardiomyocytes. Scale bars indicate (in μm) 20 (*c*), 50 (*d, e*), 10 (*f*), 5 (*g, h*), and 2 (*f*, inset). The series of experiments was performed on 6 rats.

protein connexin 43 further revealed that these stem cell-derived cardiomyocytes were integrated with the surrounding heart tissue (Fig. 6*h*). In fact, compared with sham-injected hearts (n=3), echocardiography of stem cell transplanted hearts (n=4) revealed a significantly greater left ventricular ejection fraction (**Fig. 7*a***) as derived from M-mode images (Fig. 7*b*). Whereas in sham-injected hearts the infarcted anteroseptal wall was akinetic, the corresponding area in stem cell-injected hearts displayed contractility indicative of viable myocardium (Fig. 7*c*). A positive inotropic β-adrenergic response was observed in stem cell engrafted hearts but was absent in sham-injected failing hearts (unpublished results). Thus, stem cells differentiate into functional cardiomyocytes within infarcted hearts, resulting in improved function.

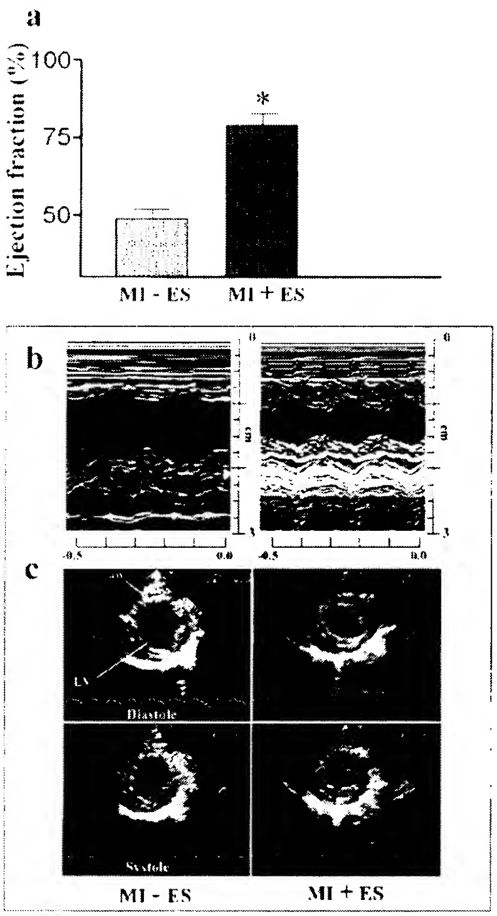

Figure 7. Gain of function of infarcted hearts after stem cell therapy. *a)* Average left ventricular ejection fraction 4 wk after sham-injection (MI-ES, n=3) or stem cell transplantation (MI+ES n=4) in rats with myocardial infarction. *Significant difference between the two groups (P<0.01). *b)* Representative M-mode scans obtained as 1-dimensional images at the greatest dimension of the left ventricular base as a function of time. Note improved mobility of the anterior wall (top of image) in the stem cell-transplanted (right) vs. the sham-injected (left) postinfarcted hearts. *c)* Two-dimension echocardiographic images through the base of the left ventricle (LV) in diastole (top) and systole (bottom). Note less dilatation and greater anterior wall (aw) contractility in the stem cell-transplanted (right) compared to the sham-injected (left) heart.

DISCUSSION

A promising approach for cardiac repair is cell-based repopulation of diseased myocardium. However, the originally used differentiated cardioblasts possess a limited mitotic aptitude, reducing their potential to replace extensive necrotic areas after ischemic injury. In this regard undifferentiated stem cells have a higher proliferative capability, and adult stem cells have recently been shown to differentiate into cardiomyocytes when engrafted in hearts before or shortly after infarction, improving cardiac function (15, 16). No data, however, are available on the potential for differentiation of stem cells engrafted into a diseased heart weeks after infarction, a more clinically relevant situation. Here we provide the first evidence that 4 wk after infarction, embryonic stem cells can extensively repopulate scar tissue within neovascularized (40) scar tissue. Ventricular specification of differentiated stem cells was proved by positive staining for MLC2v, a specific ventricular sarcomeric protein. Such cells expressed the gap junction protein connexin 43, suggesting integration and synchronization with the host tissue. This resulted in enhancement of wall motion in the infarct zone and significant improvement of ventricular contractile performance, including recovery of the β-adrenergic response. This together with the recent observation that heart injection of differentiated cardiomyocytes, but not fibroblasts, improved cardiac contractile function (41) argues in favor of a gain in function provided by contractile cardiomyocytes differentiated from ES cells. Altogether, our findings provide the first example of the potential therapeutic benefit of undifferentiated embryonic stem cells for the diseased heart.

Rejection of the stem cell graft by the host was not noted. This could be due to the absence of MHC antigen expression by stem cells (42). Stem cells can induce down-regulation of the host immune response; in fact, engraftment may be successful without immunosuppression due to induction of mixed immune chimerism favoring long-term graft acceptance (42). Here, stem cells were injected into the myocardium without coadministration of immunogenic molecules, which could explain the absence of mobilization of T cells, whose pool is likely to be modest (43).

Our findings are in line with previous work showing the propensity of embryoid bodies formed from embryonic stem cells to regenerate spinal cord when engrafted into the appropriate host environment (44). In fact, the present study provides a direct demonstration that the cardiac host environment is sufficient to commit undifferentiated embryonic stem cells with a high repertoire of potential fates toward a very specific cell lineage, namely, the cardiac ventricular myocyte. Growth and differentiation factors determine the fate of stem cells (45). TGF-β and BMP2 released by cardiac

myocytes or fibroblasts (34, 46, 47) are mandatory in early cardiogenesis in avian or *Xenopus* embryos (35, 48, 49). These factors are secreted chronically after myocardial infarction (38). Here, members of the TGF-β family induced strong expression of mesodermal as well as early and late cardiac markers, indicating their ability to mediate cardiac commitment of mammalian embryonic stem cells. TGF-β- and BMP2-treated stem cells generated embryoid bodies with extensive myofibrillar networks and, in the presence of host cells secreting a TGF-β family member, readily differentiated into cardiomyocytes with sarcomeric units similar to those of mature cardiac cells. The requirement for TGF-β and BMP2 is underscored by use of disrupters of TGF-β/BMP2 receptor-mediated signaling, LAP (37), noggin (36), and ΔKTGFβRII (22), which prevented differentiation of stem cells in vitro or in vivo. Thus, stem cells unable to respond to TGF-β/BMP2 signals remained undifferentiated and could proliferate into invasive tumors. This provides direct evidence that TGF-β superfamily members secreted by the heart (39, 50) are essential for stem cell differentiation into cardiomyocytes, thereby demonstrating a paracrine role for the heart in this process.

In principle, the molecular mechanism underlying TGF-β/BMP2 induced cardiac differentiation could be due to an 'instructive' or 'selective' modality (51). The first refers to TGF-β- and BMP2-directed cardiac differentiation and prevention of other fates, such as myogenesis (52). The second refers to cardiac differentiation induced by other conditions, such as cell–cell interaction, in which TGF-β and BMP2 favor selection through a proliferative or survival advantage (34). TGF-β and BMP2 actively up-regulated expression of cardiac transcription factors in stem cells, and the absence of TGF-β/BMP2 signaling kept stem cells undifferentiated. Only coculture of ES cells with C9 cells secreting BMP2 (but not with wild-type C3H10) cells induced their cardiac differentiation, suggesting that cell–cell interaction alone was insufficient to secure cardiac differentiation. Thus, our data rather suggest an instructive mechanism underlying a true differentiation action for TGF-β and BMP2 on stem cells.

In summary, we found that TGF-β superfamily members trigger in embryonic stem cells the expression of otherwise silent genes (Nkx2.5, MEF2C), a hallmark of cardiac phenotype, and activate cardiac promoters after stem cell engraftment. Driven by these differentiation factors, stem cell-derived cardiomyocytes integrate with the surrounding myocardium, express ventricular specific proteins, and contract in synchrony with the host. These three criteria, recently described as necessary for establishing the occurrence of a cell fate change (45), are demonstrated here. Finally, our demonstration that stem cell-derived cardiomyocytes improve contractile performance of infarcted hearts, presents the opportunity for exploitation of the paracrine TGF-β/BMP2 function of the heart in optimizing stem cell-based therapeutic strategies for cardiac repair. **FJ**

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roughly 300 animals, and given a choice between an optimal concentration of benzaldehyde (1:200 dilution in ethanol) and a lower concentration of diacetyl (1:10,000 dilution in ethanol) in the presence of a uniform field of butanone (1.2 µl per 10-ml plate). Under these conditions more than 95% of wild-type animals prefer benzaldehyde. Animals that accumulated at the diacetyl source were removed and retested under the same conditions to repeat the enrichment. Animals that preferred diacetyl three times were isolated, and their F2 broods were given a choice between benzaldehyde and diacetyl in the absence of a uniform concentration of butanone. Mutants that could chemotax to benzaldehyde under these conditions were saved. Twenty-seven mutants exhibited discrimination defects that could also be replicated without the diacetyl counterattractant. Mutants were backcrossed twice to wild-type animals.

Genetic mapping of *ky542*

We mapped *ky542* to chromosome II by observing segregation of the discrimination phenotype away from the dominant marker *qt 1(sc1)* (7/7 isolates). Subsequent mapping was performed by following segregation of the discrimination phenotype with single-nucleotide polymorphisms (SNPs) between the wild-type N2 and CB4856 strains. F2 progeny of *ky542* × CB4856 crosses were isolated, and populations were generated from each isolate. Each population was tested for butanone/benzaldehyde discrimination. Populations that were homozygous mutant and those that were homozygous wild type were retained, whereas populations that appeared to be heterozygous were discarded. We isolated DNA from each population and scored SNPs by polymerase chain reaction amplification followed by restriction enzyme digestion. Using 33 populations, we found that *ky542* mapped between SNPs located on cosmid C01F1 (chromosome II, position 1.5) and cosmid C34F1 (chromosome II, position −2.5).

Laser ablations

AWC neurons were ablated in a wild-type strain that contained an integrated *str-2::GFP* reporter (*kyIs140*) at the L1 or L2 larval stage[1]. The AWC neuron was identified by its characteristic position or by the use of the *str-2::GFP* marker, and then laser irradiated. Ablation was confirmed for AWC[ON]-ablated animals by looking for *str-2::GFP* expression after all assays had been performed. Single-animal assays were performed on gravid adults as early as the second day after ablation and as late as the fourth day. We assayed the same animals on two or three consecutive days. As many as three consecutive olfactory assays were performed in a single day. For discrimination assays, in which animals were challenged with the same attractant in the presence and absence of saturating odour, animals were allowed to recover between tests for 1 h on a fresh plate with no odorants. The order of the assays was randomized on different days. Single-animal assay plates were poured 1 day before the assays and allowed to air dry for 1 h before the assays.

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Bone marrow cells regenerate infarcted myocardium

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Myocardial infarction leads to loss of tissue and impairment of cardiac performance. The remaining myocytes are unable to reconstitute the necrotic tissue, and the post-infarcted heart deteriorates with time[1]. Injury to a target organ is sensed by distant stem cells, which migrate to the site of damage and undergo alternate stem cell differentiation[2–5]; these events promote structural and functional repair[6–8]. This high degree of stem cell plasticity prompted us to test whether dead myocardium could be restored by transplanting bone marrow cells in infarcted mice. We sorted lineage-negative (Lin⁻) bone marrow cells from transgenic mice expressing enhanced green fluorescent protein[9] by fluorescence-activated cell sorting on the basis of c-*kit* expression[10]. Shortly after coronary ligation, Lin⁻ c-*kit*^{POS} cells were injected in the contracting wall bordering the infarct. Here we report that newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. The developing tissue comprised proliferating myocytes and vascular structures. Our studies indicate that locally delivered bone marrow cells can generate *de novo* myocardium, ameliorating the outcome of coronary artery disease.

Injection of male Lin⁻c-*kit*^{POS} bone marrow cells (see Supplementary Information) in the peri-infarcted left ventricle of female mice resulted in myocardial regeneration. Repair was obtained in 12 out of 30 mice (40%). Failure to reconstitute infarcts was attributed to the difficulty of transplanting cells into tissue contracting at 600 beats per minute. However, an immunological reaction to the histocompatibility antigen on the Y chromosome of the donor bone marrow cells could account for the lack of repair in some of the female recipients. Closely packed myocytes occupied 68 ± 11% of the infarcted region and extended from the anterior to the posterior aspect of the ventricle (Fig. 1a–d). The fraction of endocardial and epicardial circumference delimiting the infarcted area[1,11] did not differ in untreated mice, 78 ± 18% ($n = 8$), or in mice treated with Lin c-*kit*^{POS} cells, 75 ± 14% ($n = 12$), or Lin c-*kit*^{NEG} cells, 75 ± 15% ($n = 11$). New myocytes were not found in mice injected with Lin c-*kit*^{NEG} cells (Fig. 1e).

The origin of the cells in the forming myocardium was deter-

mined by the expression of enhanced green fluorescent protein (EGFP) (Fig. 2; see also Supplementary Information) and the presence of Y chromosome (Supplementary Information). EGFP was restricted to the cytoplasm, whereas Y chromosome was restricted to the nuclei of new cardiac cells. EGFP and Y chromosome were not detected in the surviving portion of the ventricle. EGFP expression was combined with the labelling of proteins specific for myocytes, endothelial cells and smooth muscle cells. This allowed us to identify each cardiac cell type, and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a–c; see also Supplementary Information). The percentage of new myocytes, endothelial cells and smooth muscle cells expressing EGFP was $53 \pm 9\%$ ($n = 7$), $44 \pm 6\%$ ($n = 7$) and $49 \pm 7\%$ ($n = 7$), respectively. These values were consistent with the fraction of transplanted Lin c-*kit*^{POS} bone marrow cells that expressed EGFP, $44 \pm 10\%$ ($n = 6$). An average $54 \pm 8\%$ ($n = 6$) of myocytes, endothelial cells and smooth muscle cells expressed EGFP in the heart of donor transgenic mice.

To confirm that newly formed myocytes represented maturing cells aiming at functional competence, we examined expression of the myocyte enhancer factor 2 (MEF2), the cardiac specific transcription factor GATA-4 and the early marker of myocyte development Csx/Nkx2.5. In the heart, MEF2 proteins are recruited by GATA-4 to activate synergistically the promoters of several cardiac genes, such as myosin light chain, troponin T, troponin I, α-myosin heavy chain, desmin, atrial natriuretic factor and α-actin[12,13]. Csx/Nkx2.5 is a transcription factor restricted to the initial phases of

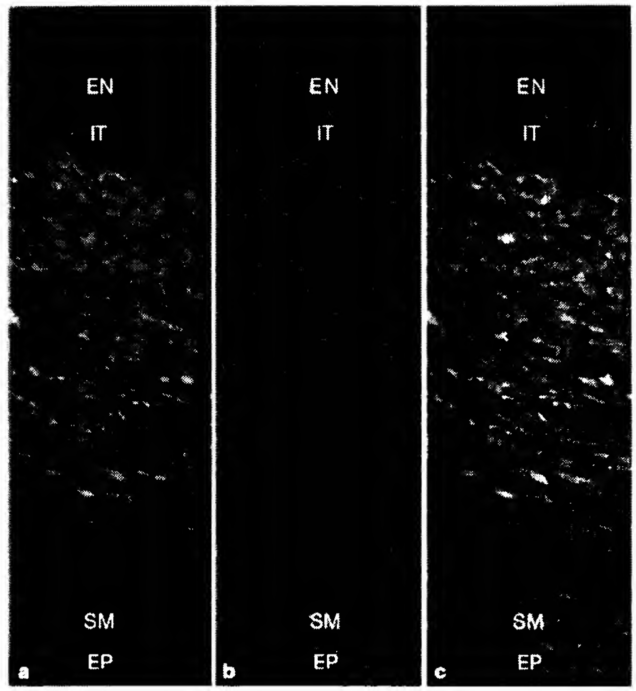

Figure 2 Myocardial infarct injected with Lin$^-$ c-*kit*^{POS} cells; myocardium is regenerating from endocardium (EN) to epicardium (EP). **a**, EGFP (green); **b**, cardiac myosin (red); **c**, combination of EGFP and myosin (red–green), and propidium-iodide-stained nuclei (blue). Infarcted tissue (IT) can be seen in the subendocardium, spared myocytes (SM) can be seen in the subepicardium. Original magnification, ×250 (**a–c**).

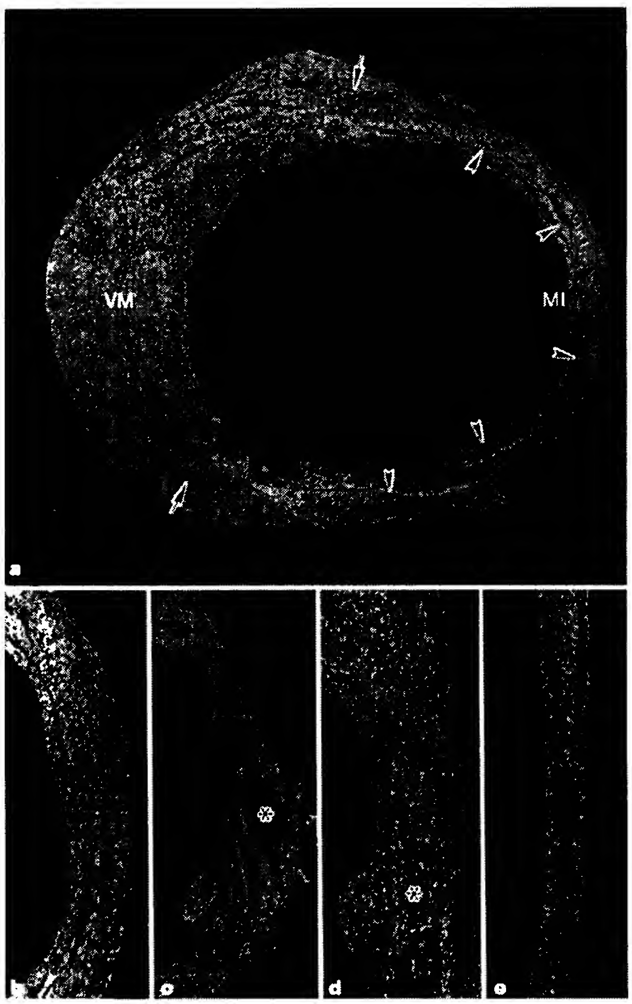

Figure 1 Bone marrow cells and myocardial regeneration. **a**, Myocardial infarct (MI) injected with Lin$^-$ c-*kit*^{POS} cells from bone marrow (arrows). Arrowheads indicate regenerating myocardium; VM, viable myocardium. **b**, Same MI at higher magnification. **c**, **d**, Low and high magnifications of MI injected with Lin$^-$ c-*kit*^{POS} cells. **e**, MI injected with Lin$^-$ c-*kit*^{NEG} cells; only healing is apparent. Asterisk indicates necrotic myocytes. Red, cardiac myosin; green, propidium iodide labelling of nuclei. Original magnification, ×12 (**a**); ×25 (**c**) ×50 (**b**, **d**, **e**).

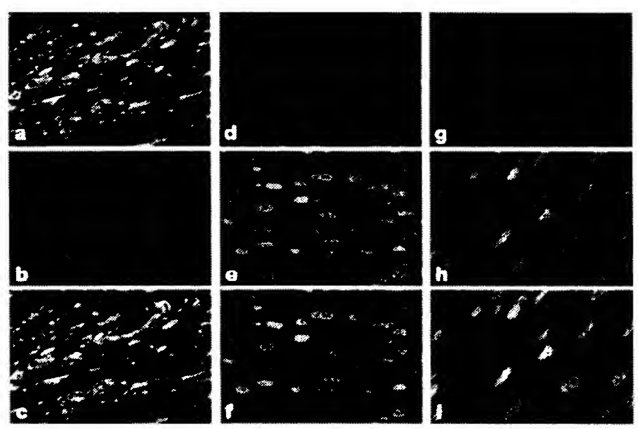

Figure 3 Regenerating myocardium in myocardial infarct injected with Lin$^-$ c-*kit*^{POS} cells. **a**, EGFP (green); **b**, smooth muscle α-actin in arterioles (red); **c**, combination of EGFP and smooth muscle α-actin (yellow–red), and propidium iodide (PI)-stained nuclei (blue). **d–i**, MEF2 and Csx/Nkx2.5 in cardiac myosin-positive cells. **d**, **g**, PI-stained nuclei (blue); **e**, **h**, MEF2 and Csx/Nkx2.5 labelling (green); **f**, **i**, cardiac myosin (red), and combination of MEF2 or Csx/Nkx2.5 with PI (bright fluorescence in nuclei). Original magnification, ×300 (**a–i**).

myocyte differentiation[12]. In the reconstituting heart, all nuclei of cells labelled with cardiac myosin expressed MEF2 (Fig. 3d–f) and GATA-4 (Supplementary Information), but only 40 ± 9% expressed Csx/Nkx2.5 (Fig 3g–i).

To characterize further the properties of these myocytes, we determined the expression of connexin 43. This protein is responsible for intercellular connections and electrical coupling through the generation of plasma-membrane channels between myocytes[14,15]; connexin 43 was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells (Fig. 4). These results were consistent with the expected functional competence of the heart muscle phenotype. In addition, myocytes at various stages of maturation were detected within the same and different bands (Fig. 5).

Ki67 is expressed in cycling cells in G1, S, G2 and early mitosis[16], providing a quantitative estimate of the fraction of cells in the cell cycle at the time of observation. 5-Bromodeoxyuridine (BrdU) labelling identifies nuclei in S phase[16,17]; therefore, we injected BrdU for 4–5 days to assess cumulative cell division during active growth (Supplementary Information). Proliferation of myocytes was 93% ($P < 0.001$) and 60% ($P < 0.001$) higher than that of endothelial cells, and 225% ($P < 0.001$) and 176% ($P < 0.001$) higher than that of smooth muscle cells, when measured by BrdU and Ki67, respectively (BrdU: myocytes 36 ± 8%; endothelial cells 19 ± 5%; smooth muscle cells 11 ± 2%; Ki67: myocytes 19 ± 3%; endothelial cells 12 ± 3%; smooth muscle cells 7 ± 2%; $n = 8$ in all cases). Dividing myocytes were small with partially aligned myofibrils, resembling late fetal/neonatal cells; 40–50% of the Ki67- or BrdU-positive cells expressed EGFP.

Cell differentiation caused a loss of c-*kit* surface receptors. We observed only two undifferentiated cells showing c-*kit* on the cell membrane in the subendocardium of the infarcted wall. These c-*kit*-labelled cells were in proximity but not within the regenerating band. They expressed EGFP, confirming their origin from the transplant (Supplementary Information).

To determine whether developing myocytes derived from the Lin⁻ c-*kit*^{POS} cells had an impact on function, we obtained haemodynamic parameters before death. Results from infarcted mice non-injected or injected with Lin⁻ c-*kit*^{NEG} cells were combined. In comparison with sham-operated mice, the infarcted groups exhibited indices of cardiac failure (Fig. 6a). In mice treated with Lin⁻ c-*kit*^{POS} cells, left ventricular (LV) end-diastolic pressure (LVEDP) was 36% lower, and developed pressure (LVDP), LV + dP/dt and LV − dP/dt were 32%, 40% and 41% higher, respectively.

Locally transplanted Lin⁻ c-*kit*^{POS} bone marrow cells have a high capacity for cardiac tissue differentiation. Here, they led to the formation of new myocytes, endothelial cells and smooth muscle cells generating *de novo* myocardium, inclusive of coronary arteries, arterioles and capillaries. The partial repair of the infarcted heart implies that the transplanted cells responded to signals from the injured myocardium that promoted their migration, proliferation and differentiation within the necrotic area of the ventricular wall (Fig. 6b). These differentiating myocytes expressed nuclear and cytoplasmic proteins typical of cardiac tissue. The presence of connexin 43 points to cellular coupling and functional competence of the restored myocardium (Fig. 6b). With postnatal maturation, stem cell function was assumed previously to be restricted to cell lineages present in the organ from which they are derived. However, this limitation in stem cell differentiation potential has been challenged by studies showing that bone marrow and neural stem cells can produce many cell types[1,5,18–20]. We report, for the first time, that a subpopulation of primitive bone marrow cells regenerate myocardium *in vivo*, replacing dead tissue.

Haematopoietic stem cells (HSCs), neural-crest-derived melanoblasts and primordial germ cells express c-*kit* on their cell membrane. These primitive cells migrate during fetal development,

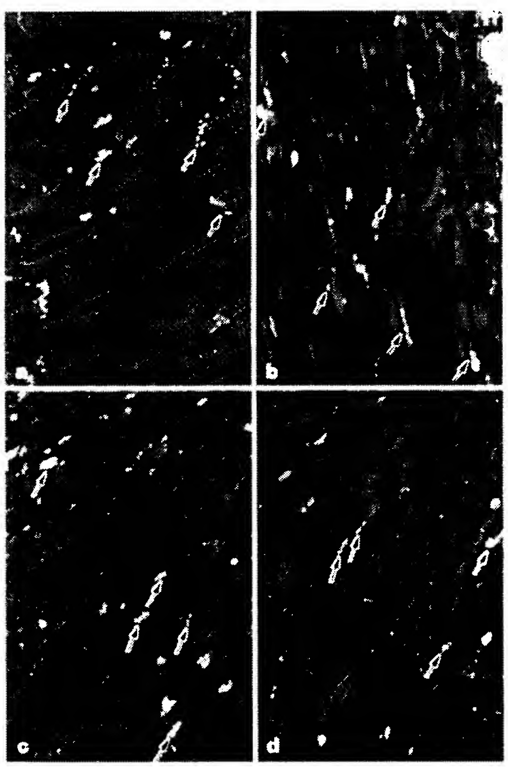

Figure 4 Myocardial repair and connexin 43. **a**, Border zone; **b–d**, regenerating myocardium. Shown are connexin 43 (yellow–green; arrows indicate contacts between myocytes) and α-sarcomeric actin (red), and PI-stained nuclei (blue). Original magnification, ×500 (**a**), ×800 (**b–d**).

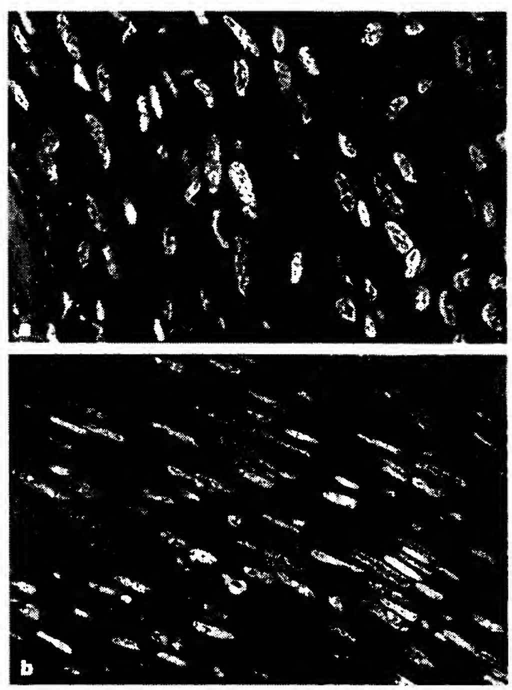

Figure 5 Myocardial infarcts injected with Lin⁻ c-*kit*^{POS} cells: regenerating myocytes. Shown are cardiac myosin (red), and propidium-iodide-labelled nuclei (yellow–green). Original magnification, ×1,000 (**a**); ×700 (**b**).

homing to the yolk sac and liver. Both of these organs are positive for messenger RNA encoding stem cell factor (SCF), the ligand for c-kit[21]. It is thought that membrane-bound SCF mediates the migration of HSC and other primitive cells to their target organs[22]. The fetal and neonatal hearts are positive for SCF transcripts[21] and, although it is not clear whether adult heart cells generate SCF, the c-kit/SCF pathway might be the mechanism by which, in our conditions, transplanted Lin$^-$ c-kit^{POS} cells migrated from the site of injection to the infarcted myocardium.

When a stem cell divides, two daughter cells are formed; these may maintain stem cell properties or become differentiating cells[23] that multiply much more rapidly than stem cells[24]. The Lin$^-$ c-kit^{POS} cells in these transplants produced the three main cell types of the heart: myocytes constituted the predominant and most active growth component of the regenerating myocardium; endothelial and smooth muscle cells were fast growing but were smaller fractions of the developing tissue. Our observations are difficult to compare with those obtained in the cryo-injured rat heart after injecting cultured myocytes derived from mesenchymal bone marrow cells[25]. Formation of myotubules *in vitro* was required for successful transplantation in that study[25], which contrasts with our results. Cryo-injury has no human counterpart. It constitutes an unusual damage with an intact coronary circulation. This may be why only a few endothelial cells were possibly linked to the original culture system[25] and smooth muscle cells were not detected. Also at variance with our data is the fact that there was no replacement of damaged myocardium with functioning tissue.

Coronary heart disease accounts for 50% of all cardiovascular deaths and nearly 40% of the incidence of heart failure. The current findings have provided compelling evidence that our approach has relevant implications for human disease. Locally delivered primitive bone marrow cells promoted successful treatment of large myocardial infarcts after the completion of ischaemic cell death. This therapeutic intervention reduced the infarcted area and improved cardiac haemodynamics. Infarct size is a major determinant of morbidity and mortality, as massive infarcts affecting 40% or more of the left ventricle in patients are associated with intractable cardiogenic shock or the rapid development of congestive heart failure[1]. In the past, recovery of cardiac function has been fully dependent on the growth of the remaining non-infarcted portion of the ventricle. However, the hypertrophied infarcted heart undergoes progressive deterioration, leading to a dilated myopathy, terminal failure and death[1]. Transplanted Lin$^-$ c-kit^{POS} bone marrow cells have the capability of regenerating acutely significant amounts of contracting myocardium. This new form of repair can improve the immediate and long-term outcome of ischaemic cardiomyopathy. □

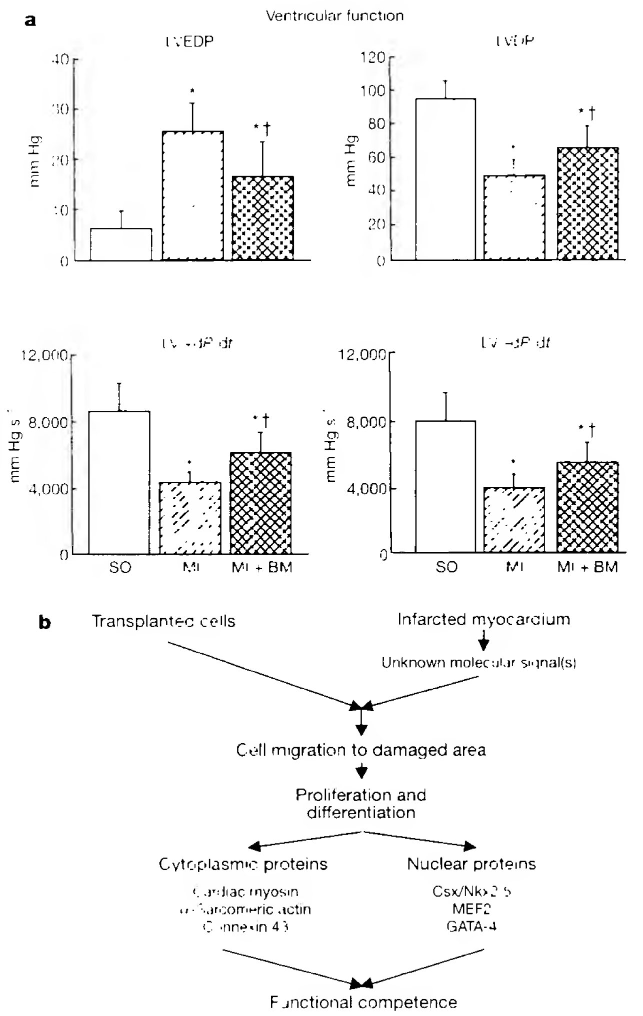

Figure 6 Postulated mechanism of myocardial regeneration and its effect on ventricular function. **a**, Effects of myocardial infarction (MI) on left ventricular end-diastolic pressure (LVEDP), developed pressure (LVDP), LV+ dP/dt (rate of pressure rise) and LV− dP/dt (rate of pressure decay). Results are from sham-operated mice (SO, $n = 11$), mice non-injected with Lin$^-$ c-kit^{POS} cells (MI; $n = 5$ injected with Lin$^-$ c-kit^{NEG} cells; $n = 6$ non-injected), and mice injected with Lin$^-$ c-kit^{POS} cells (MI+BM, $n =$ [illegible]). Values are mean ± s.d. $*P < 0.05$ versus SO; $\dagger P < 0.05$ versus MI. **b**, Proposed scheme for Lin$^-$ c-kit^{POS} cell differentiation in cardiac muscle and functional implications.

Methods

Lin$^-$ c-*kit*^{POS} cells

We collected bone marrow from the femurs and tibias of male transgenic mice expressing EGFP[7]. Cells were suspended in PBS containing 5% fetal calf serum (FCS) and incubated on ice with rat anti-mouse monoclonal antibodies specific for the following haematopoietic lineages: CD4 and CD8 (T lymphocytes), B-220 (B lymphocytes), Mac-1 (macrophages), GR-1 (granulocytes) (all Caltag Laboratories) and TER-119 (erythrocytes) (Pharmingen). Cells were then rinsed in PBS and incubated for 30 min with magnetic beads coated with goat anti-rat immunoglobulin (Polysciences). Lineage-positive cells were removed by a biomagnet and the 10% remaining lineage-negative (Lin$^-$) cells were stained with ACK-4-biotin (anti-c-*kit* monoclonal antibody). Cells were rinsed in PBS, stained with streptavidin-conjugated phycoerythrin (SA-PE) (Caltag) and sorted by FACS using a FACSVantage instrument (Becton Dickinson). We excited EGFP and ACK-4-biotin-SA-PE at a wavelength of 488 nm. The Lin$^-$ cells were sorted as c-*kit*-positive (c-kit^{POS}) and c-*kit*-negative (c-kit^{NEG}) with a 1–2 log difference in staining intensity. The c-kit^{POS} cells were suspended at a concentration of 3×10^4 to 2×10^5 cells in 5 μl of PBS, and the c-kit^{NEG} cells were suspended at a concentration of 5×10^4 to 5×10^5 in 5 μl of PBS[10].

Myocardial infarction

Myocardial infarction was induced in female C57BL/6 mice at 2 months of age as described[11]; 3–5 h after infarction, the thorax was re-opened and 2.5 μl PBS containing Lin$^-$ c-kit^{POS} cells were injected in the anterior and posterior aspects of the viable myocardium bordering the infarct. Infarcted mice that were not injected or injected with Lin$^-$ c-kit^{NEG} cells and sham-operated mice were used as controls. All animals were killed 9 ± 2 days after surgery. Protocols were approved by an institutional review board.

Ventricular function

Mice were anaesthetized with chloral hydrate (400 mg per kg (body weight), intraperitoneally (i.p.)), and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671; Millar) for the measurements of LV pressures, and LV + and LV − dP/dt in the closed-chest preparation. The abdominal aorta was cannulated, the heart was arrested in diastole, and the myocardium was perfused retrogradely with 10% buffered formalin[11,26]. Three tissue sections, from the base to the apex of the left ventricle, were stained with haematoxylin and eosin. At 9 ± 2 days after coronary occlusion, the infarcted portion of the ventricle was easily identifiable grossly and histologically (see Fig. 1a). The lengths of the endocardial and epicardial surfaces delimiting the infarcted region, and the endocardium and epicardium of the entire left ventricle, were measured in each section. Subsequently, their quotients were computed to yield the average infarct size in each case. This was accomplished at ×4 magnification with an image analyser connected to a microscope[11].

Cell proliferation and EGFP detection

To establish whether Lin⁻ c-*kit*^{POS} cells resulted in myocardial regeneration, we administered BrdU (50 mg per kg (body weight), i.p.) to the animals daily for 4–5 consecutive days before death. Sections were incubated with anti-BrdU antibody, and BrdU labelling of cardiac cells was measured[1]. Moreover, expression of Ki67 in nuclei was evaluated by treating samples with a rabbit polyclonal anti-mouse Ki67 antibody (Dako). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was used as secondary antibody. EGFP was detected with a rabbit polyclonal anti-GFP (Molecular Probes). Myocytes were recognized with a mouse monoclonal anti-cardiac myosin heavy chain (MAB 1552; Chemicon) or a mouse monoclonal anti-sarcomeric α-actin (clone 5C5; Sigma), endothelial cells with a rabbit polyclonal anti-human factor VIII (Sigma) and smooth muscle cells with a mouse monoclonal anti-smooth-muscle α-actin (clone 1A4; Sigma). Nuclei were stained with propidium iodide, 10 μg ml^{-1} (refs 27, 28). We determined the percentages of myocyte (M), endothelial cell (EC) and smooth muscle cell (SMC) nuclei labelled by BrdU and Ki67 by confocal microscopy. This was accomplished by dividing the number of nuclei labelled by the total number of nuclei examined. Numbers of nuclei sampled in each cell population were as follows. BrdU labelling: M, 2,908; EC, 2,153; SMC, 1,877. Ki67 labelling: M, 3,771; EC, 4,051; SMC, 4,752. Numbers of cells counted for EGFP labelling: M, 3,278; EC, 2,156; SMC, 1,274. We determined the percentage of myocytes in the regenerating myocardium by delineating the area occupied by cardiac-myosin-stained cells and dividing this by the total area represented by the infarcted region in each case.

Y chromosome

For the fluorescence *in situ* hybridization assay, we exposed sections to a denaturing solution containing 70% formamide. After dehydration with ethanol, sections were hybridized with the DNA probe CEP Y (satellite III) Spectrum Green (Vysis) for 3 h (ref. 29). Nuclei were stained with propidium iodide.

Transcription factors and connexin 43

Sections were incubated with rabbit polyclonal anti-MEF2 (C-21; Santa Cruz), rabbit polyclonal anti-GATA-4 (H-112; Santa Cruz), rabbit polyclonal anti-Csx/Nkx2.5 (obtained from Dr S. Izumo) and rabbit polyclonal anti-connexin 43 (Sigma). We used FITC-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody[30].

Statistical analysis

Results are presented as means ± s.d. Significance between two measurements was determined by Student's *t*-test, and in multiple comparisons was evaluated by the Bonferroni method. Values of $P < 0.05$ were considered significant.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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CaT1 manifests the pore properties of the calcium-release-activated calcium channel

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The calcium-release-activated Ca^{2+} channel, I_{CRAC}[1–3], is a highly Ca^{2+}-selective ion channel that is activated on depletion of either intracellular Ca^{2+} levels or intracellular Ca^{2+} stores. The unique gating of I_{CRAC} has made it a favourite target of investigation for new signal transduction mechanisms; however, without molecular identification of the channel protein, such studies have been inconclusive. Here we show that the protein CaT1 (ref. 4), which has six membrane-spanning domains, exhibits the unique biophysical properties of I_{CRAC} when expressed in mammalian cells. Like I_{CRAC}, expressed CaT1 protein is Ca^{2+} selective, activated by a reduction in intracellular Ca^{2+} concentration, and inactivated by higher intracellular concentrations of Ca^{2+}. The channel is indistinguishable from I_{CRAC} in the following features: sequence of selectivity to divalent cations; an anomalous mole fraction effect; whole-cell current kinetics; block by lanthanum; loss of selectivity in the absence of divalent cations; and single-channel conductance to Na^+ in divalent-ion-free conditions. CaT1 is activated by both passive and active depletion of calcium stores. We propose that CaT1 comprises all or part of the I_{CRAC} pore.

Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells

See related Commentary on pages 1355–1356.

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Myocyte loss in the ischemically injured mammalian heart often leads to irreversible deficits in cardiac function. To identify a source of stem cells capable of restoring damaged cardiac tissue, we transplanted highly enriched hematopoietic stem cells, the so-called side population (SP) cells, into lethally irradiated mice subsequently rendered ischemic by coronary artery occlusion for 60 minutes followed by reperfusion. The engrafted SP cells ($CD34^-$/low, $c\text{-}Kit^+$, $Sca\text{-}1^+$) or their progeny migrated into ischemic cardiac muscle and blood vessels, differentiated to cardiomyocytes and endothelial cells, and contributed to the formation of functional tissue. SP cells were purified from Rosa26 transgenic mice, which express lacZ widely. Donor-derived cardiomyocytes were found primarily in the peri-infarct region at a prevalence of around 0.02% and were identified by expression of lacZ and α-actinin, and lack of expression of CD45. Donor-derived endothelial cells were identified by expression of lacZ and Flt-1, an endothelial marker shown to be absent on SP cells. Endothelial engraftment was found at a prevalence of around 3.3%, primarily in small vessels adjacent to the infarct. Our results demonstrate the cardiomyogenic potential of hematopoietic stem cells and suggest a therapeutic strategy that eventually could benefit patients with myocardial infarction.

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Introduction

Occlusion of a coronary vessel and the resultant myocardial ischemia rapidly results in myocardial necrosis followed by scar formation. When the ischemic myocardium is reperfused, there is a rapid onset of contraction band necrosis and an intense inflammatory cascade. Inflammatory cells, although potentially injurious, are critical to efficient repair of the ventricular wall, mediating a process that involves resorption of necrotic material, scar formation, and angiogenesis (1). While efficient repair will allow the ventricle to function despite the loss of some of its cardiac myocytes (contractile subunits), it has been well established that adult cardiac myocytes do not replicate, thus these pump units are not actually replaced. Thus, microvascular repair can ensue through replication of smooth muscle cells and endothelial cells, but the ventricular myocytes are replaced by scar. The recent progress in the area of stem cell research has led to the suggestion that primitive stem cells might potentially be used to regenerate cells in organs in which no parenchymal regeneration occurs. The purpose of this study was to examine the possibility that primitive stem cells might contribute to regeneration in the infarcted myocardium

Recent research suggests that primitive stem cells within whole bone marrow possess greater functional plasticity than was suspected previously. After bone marrow transplantation, donor-derived stem cells have been found in such diverse nonhematopoietic tissues as skeletal muscle (2), cardiac muscle (3), liver bile ducts (4, 5), and vascular endothelium (6–9). The stem cell compartment in human bone marrow is highly complex, comprising both $CD34^+$ and $CD34^-$ hematopoietic stem cells, mesenchymal progenitors, and perhaps other cell types whose activities remain to be defined. In the study reported here, we tested a novel "side population" (SP) of $CD34^-$ stem cells, selected on the basis of Hoechst dye staining, for their capacity to regenerate cardiac myofibers and blood vessels in ischemically injured cardiac tissue. SP cells have been well characterized previously and shown to be potent hematopoietic stem cells (10, 11) capable of contributing to regeneration of skeletal muscle (12). In the present study, SP cells have been more thoroughly characterized by RT-PCR and Ab staining in order to bet-

ter define the differentiation potential of these transplanted stem cells. After their engraftment in the ischemically injured hosts, SP cells or their progeny became incorporated into both cardiac muscle and vessel structures, where they displayed the characteristics of differentiated cardiomyocytes as well as endothelial cells.

Methods

Isolation of SP cells. Bone marrow specimens extracted from the tibias and femurs of C57BL/6-Rosa (a mixture of homozygous and heterozygous animals) and C57BL/6-Ly-5.1 mice 6 to 12 weeks of age were suspended at 10^6 cells/ml in DMEM supplemented with 2% FCS/10mM HEPES (HyClone Inc., Logan, Utah, USA, and Life Technologies Inc., Carlsbad, California, USA, respectively) and stained with 5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, USA) for 90 minutes at 37°C, as described previously (10, 13). The cells were then resuspended in cold HBSS containing 2% FCS and 2 μg/ml propidium iodide.

SP cells, selected as shown in Figure 1, typically accounted for 0.05% of whole bone marrow. These stem cells were additionally tested for c-Kit expression with an anti-c-Kit Ab (2B8; PharMingen, San Diego, California, USA) and for PECAM-1 (CD31) expression with anti-CD31-biotin (MEC 13,3; PharMingen) followed by staining with streptavidin-phycoerythrin (streptavidin-PE) (Molecular Probes Inc., Eugene, Oregon, USA). Expression of the Tie-2 protein was determined by Hoechst 33342 staining followed by fluorescein di-β-D-galactopyranoside (FDG) staining (Molecular Probes Inc.) of whole bone marrow from mice expressing the *lacZ* gene under control of the *Tie-2* promoter, FVB/N-TgN(TIE2LacZ)182 Sato (The Jackson Laboratory, Bar Harbor, Maine, USA) (14). Sorting and analysis of SP cells were performed on a triple-laser instrument (MoFlow; Cytomation Inc., Fort Collins, Colorado, USA). An argon laser tuned to 350-nm emission was used to excite the Hoechst dye. Fluorescence emission was collected with a 405/30 band pass (BP) filter (Hoechst blue) and a 670/40 BP filter (Hoechst red). A second 488-nm argon laser was used to excite PE and FITC. The purity of SP cells in the sorted samples was routinely greater than 91%.

RNA extraction and RT-PCR analysis. Total RNA was extracted from purified SP cells from C57BL/6-Ly-5.1 mice using Tri-Reagent (Sigma-Aldrich). Approximately, 100 ng of total RNA was treated with DNAase (Life Technologies Inc.) and used in a first-strand reaction that included oligo dT primers and Superscript reverse transcriptase (Life Technologies Inc.). Nested PCR was performed on cDNA under the following conditions: 94°C 1 minute, 60°C 1 minute, 72°C 1 minute for 35 cycles. Five microliters of the first PCR reaction was used as a template in the nested PCR reaction. The following primers were used: *angiopoietin-1* (580 bp), 5′-CAGTGGCTG-CAAAAACTTGA-3′ forward, 5′-TCTGCACAGTCTC-GAAATGG-3′ reverse; *angiopoietin-2* (666 bp), 5′-CACACTGACCTTCCCCAACT-3′ forward, 5′-TGGT-

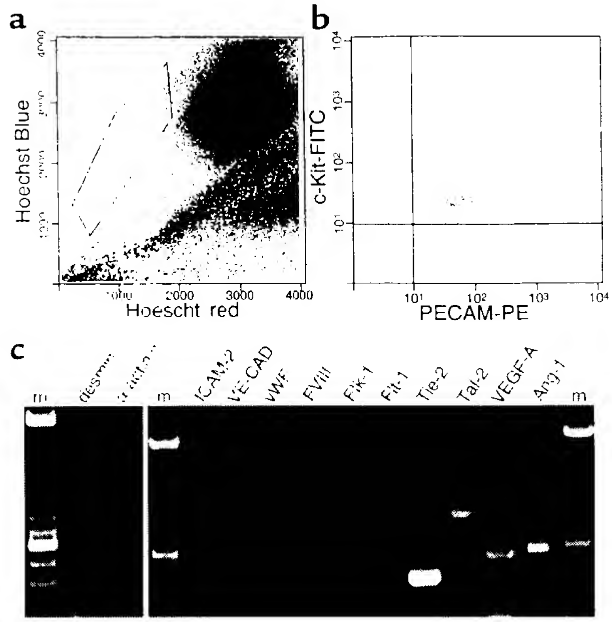


Figure 1
Gene-expression analysis of bone marrow SP cells. (**a**) Normal murine bone marrow is stained with Hoechst 33342. The indicated SP population comprises around 0.05% of total bone marrow cells. (**b**) The majority of SP cells are positive for the markers c-Kit and PECAM-1. (**c**) RT-PCR analysis of purified SP cells. m, marker; VE-CAD, VE-cadherin, FVIII, factor VIII.

GTCTCTCAGTGCCTTG-3′ reverse; *Tal-1* (984 bp), 5′-GTCCTCACACCAAAGTAGTG-3′ forward, 5′-AAAC-TAAGCAAGAATGAGATC-3′ reverse; *Tie-1* (300 bp), 5′-ACCCACTACCAGCTGGATGT-3′ forward, 5′-ATCGT-GTGCTAGCATTGAGG-3′ reverse, *Tie-2* (414 bp), 5′-CCTTCCTACCTGCTA-3′ forward-1, 5′-CCGTGGACAG-GGGAGATAAT-3′ forward-2, 5′-CCACTACACCT-TTCTTTACA-3′ reverse; *ICAM-2* (320 bp), 5′-CATATGGTCCGAGAAGCAGA-3′ forward, 5′-TGCACTCAATGGTGAAGTCT-3′ reverse; *VE-cadherin* (520 bp), 5′-TTGCCCAGCCCTAGCAACCTAAAG-3′ forward, 5′-ACCAC-CGCCCTCCTCATCGTAAGT-3′ reverse; *vWF* (1097 bp), 5′-ATGATGGAGAGGTTACACATC forward-1, 5′-GCGCATCCGCGTGGCAGTGG-3′ forward-2, 5′-GGCAGTTGCAGACCCTCCTTG-3′ reverse; *FVIII* (400 bp), 5′-GTCCCTACTCCTTCTATTCTAGCC-3′ forward-1, 5′-CTTCGCATGGAGTTGATGGGCTGT-3′ forward-2, TCATCATAGGTGTGGATGAGTCCTG-3′ reverse; *VEGF-A* (620, 548, 488 bp), 5′-GGATCCATGAACTTTCTGCT-3′ forward-1, 5′-GGGTGCACTGGACCCTGGCT-3′ forward-2, 5′-GAATTCACCGCCTCGGCTTGTC-3′ reverse; *Flk-1* (537 bp), 5′-GCCAATGAAGGGGAACTGAAGAC-3′ forward, 5′-TCTGACTGCTGGTGATGCTGTC-3′ reverse; *Flt-1* (504 bp), 5′-TGTGGAGAAACTTGGTGACCT-3′ forward, 5′-TGGAGAACAGCAGGACTCCTT-3′ reverse; smooth muscle α-actin (240 bp), 5′-GAGAAGC-CCAGCCAGTCG-3′ forward, 5′-CTCTTGCTC-TGGGCTTCA-3′ reverse; *calponin* (213 bp), 5′-CACCAACAAGTTTGCCAG-3′ forward, 5′-TGT-

GTCGCAGTGTTCCAT-3′ reverse; *desmin* (377 bp), 5′-ATGAGCCAGGCCTACTCGTC-3′ forward, 5′-GCGCACCTTCTCGATGTAGT-3′ reverse; *α-actinin* (976 bp), 5′-TGCTGCTATGGTGTCAGAGG-3′ forward, 5′-CCGATCATTGACGTTCACAG-3′ reverse.

Bone marrow transplantation and cardiac occlusion. Female C57BL/6-Ly-5.1 mice were irradiated (9 Gy) and injected with 2,000 SP cells isolated from male C57BL/6-Rosa-Ly-5.2 mice. Engraftment, which ranged from 35–85%, was determined by analysis of peripheral blood using Abs against Ly-5.2 (clone 104; PharMingen) or FDG staining for lacZ expression (Molecular Probes Inc.) followed by FACS analysis. Approximately 10 weeks after bone marrow transplantation, the left anterior descending coronary artery of each mouse was occluded for 60 minutes, followed by reperfusion as described previously (15). Two or four weeks after cardiac injury, hearts were removed and frozen for immunohistochemical analysis. At 2 and 4 weeks, the survival rate was 26%. Fifty-three percent of the mice survived at least 1 week, and 80% survived at least 24 hours of reperfusion after the 1 hour occlusion, consistent with previous observations.

Immunohistochemical analysis. Frozen sections (12 μM) of experimental tissue were fixed with 4% paraformaldehyde for 30 minutes at 4°C. LacZ staining was performed overnight at 37°C with the following reagents: 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 (NP-40), and 1 mg/ml X-gal (Life Technologies Inc.). The tissues were then washed in PBS and stained with specific Ab's. Anti-α-actinin (clone EA-53; Sigma-Aldrich) was used to identify cardiac muscle, while anti-Flt-1 (clone C-17; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and anti-ICAM-1 (generously provided by Alan Burns, Baylor College of Medicine) were used to recognize endothelial cells. Hematopoietic cells were visualized with anti-Ly-5-biotin (clone 30-F11; PharMingen). Secondary Ab's were conjugated to Alexa 488, Alexa 546, or Alexa 594 (all from Molecular Probes Inc.). Slides of the stained tissues were prepared and analyzed by fluorescence and differential interference contrast microscopy. Some sections were costained with the antimacrophage Ab, F480 (Serotec Ltd., Oxford, United Kingdom), which was detected with a kit from Vector Laboratories (Burlingame, California, USA) comprising a stain with a biotinylated anti-rat secondary followed by an avidin-biotin complex developed with 3,3-diaminobenzidine (DAB) from Vector Labs; the counterstain was eosin.

Results

The SP of murine bone marrow stem cells selected by Hoechst dye staining (Figure 1a) is a highly enriched hematopoietic stem cell population that can give rise to all hematopoietic lineages in the mouse (10, 11). To demonstrate a contribution from SP cells to the repair of nonhematopoietic tissues, we first tested for the expression of markers of primitive and fully differentiated endothelial cells and cardiomyocytes. As shown in Figure 1b, the intrinsically c-Kit–positive SP cells also expressed PECAM-1 (CD31), which was thought previously to be restricted to angioblasts, endothelial cells, megakaryocytes, and platelets. RT-PCR analysis of RNA from purified SP cells (Figure 1c) failed to detect common markers of cardiac muscle (*desmin* and *α-actinin*), differentiated vascular endothelial cells (*ICAM-2*, *VE-cadherin*, *vWF*, and *factor VIII*), and early endothelial progenitor cells (*Flk-1* and *Flt-1*, the receptors for VEGF). However, the SP cells did express the *Tie-2* gene, which encodes a receptor for angiopoietins 1 and 2. This finding was confirmed by examining the expression of lacZ in SP cells from a mouse that expressed this gene under control of the *Tie-2* promoter/enhancer (data not shown) (14, 16). The purified SP cells also expressed the early hematopoietic/endothelial cell transcription factor *Tal-1/SCL*, as well as three isoforms of *VEGF-A* and angiopoietin-1 (Ang-1). In all instances of marker positivity, the RT-PCR signal was present in three of three individual preparations of purified SP cells. The identities of the PCR products were confirmed by DNA sequencing.

To assess the contribution of SP cells to the repair of injured heart muscle, we employed bone marrow transplantation and a model of cardiac occlusion/reperfusion (Figure 2). Twenty-two C57Bl/6 mice were lethally irradiated and transplanted with SP cells purified from the bone marrow of 6-to-8-week-old adult C57Bl/6-Rosa26 mice, a strain in which the *lacZ* gene is expressed widely (17). Ten to 12 weeks after transplantation, the

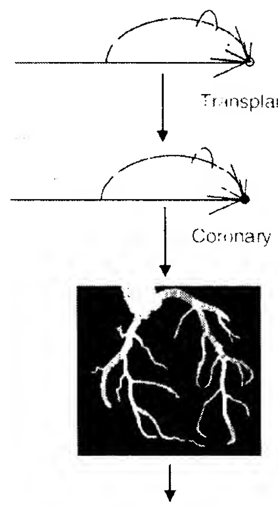
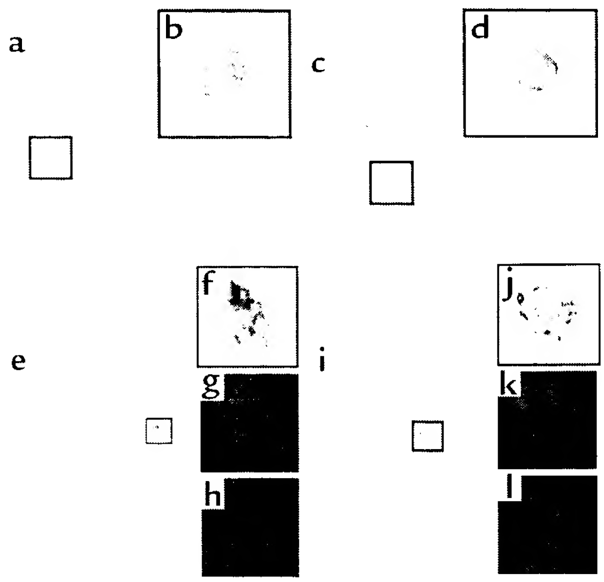


Figure 2

Experimental schema. SP cells were purified from bone marrow (BM-SP) obtained from C57Bl/6-Rosa26 transgenic mice. These cells were transplanted into lethally irradiated recipients. After 2 months, stable engraftment was determined by establishing the presence of lacZ-positive peripheral blood cells. Highly engrafted animals were subjected to coronary artery ligation for 60 minutes, followed by reperfusion. Two to four weeks later, the surviving animals were sacrificed for analysis of lacZ incorporation into cardiac tissue.

Figure 3
Incorporation of SP cells into vascular endothelial cells. (**a**–**d**) X-gal-stained section of cardiac tissue from an infarcted SP cell-transplant recipient; **b** and **d** show magnifications of the indicated capillaries from **a** and **c**. (**e**–**l**) Cardiac tissue as above, stained for expression of endothelial markers. An enlargement of the vessel stained for lacZ in **e** is seen in **f**. The same vessel is shown in **g**, stained with Flt-1, and in **h**, with ICAM-1. An enlargement of the vessel stained for lacZ in **i** is seen in **j**. The same vessel is shown in **k**, stained for Flt1, and in **l**, for ICAM1. **a**, **c**, **e**, and **i**: ×200; **b**, **d**, **f**–**h**, and **j**–**l**: ×1,000.



left anterior descending coronary artery was occluded for 1 hour and reperfused. Nineteen mice were treated, and sham operations were performed on three animals. Mice were sacrificed 2 or 4 weeks after injury in order to analyze hearts for incorporation of SP cells or their progeny into regenerating tissues. Five mice (26%) that underwent occlusion/reperfusion of the coronary artery survived to the end of the experiment, a mortality rate consistent with previous observations (15).

The hearts of mice that survived for 2 or 4 weeks after injury were sectioned, stained with X-gal, followed by fluorescent Ab's, and scanned for incorporation of transplanted cells into regenerating tissues. We observed lacZ staining in vessel structures of various caliber, but most commonly in capillaries (Figure 3, a–d). The lacZ-positive cells costained with Ab's against Flt-1 and ICAM-1 (Figure 3, e–l), demonstrating that SP cells or their progeny had migrated to the injured heart via the circulation, localized to newly forming vessels, and appeared to have integrated into the surface lining as differentiated endothelial cells. Analyses of SP-cell incorporation into specific vessel structures (i.e., arterioles, venules, lymphatic vessels) is ongoing. We are also further investigating the relative contribution of SP cells to the regeneration of specific vascular cell types (endothelial, pericyte, and smooth muscle cells). We found no lacZ-positive cells in vessel structures of sham-treated animals.

We also observed incorporation of lacZ-positive cells into cardiac muscle (Figure 4). In contrast to the sham-treated control, which lacked any evidence of SP-cell recruitment, cardiac tissue from the treated mice showed blue staining throughout the heart (Figure 4, c–h). Although the extent of staining varied appreciably, myocardial tissue specimens from all transplant recipients were lacZ positive. In some cases, particularly in animal 6, which had a remarkably high level of lacZ positivity, the staining extended throughout large tracts (Figure 4, c–e). In many cases, the staining was in a punctate pattern (Figure 4g). Cardiac muscle

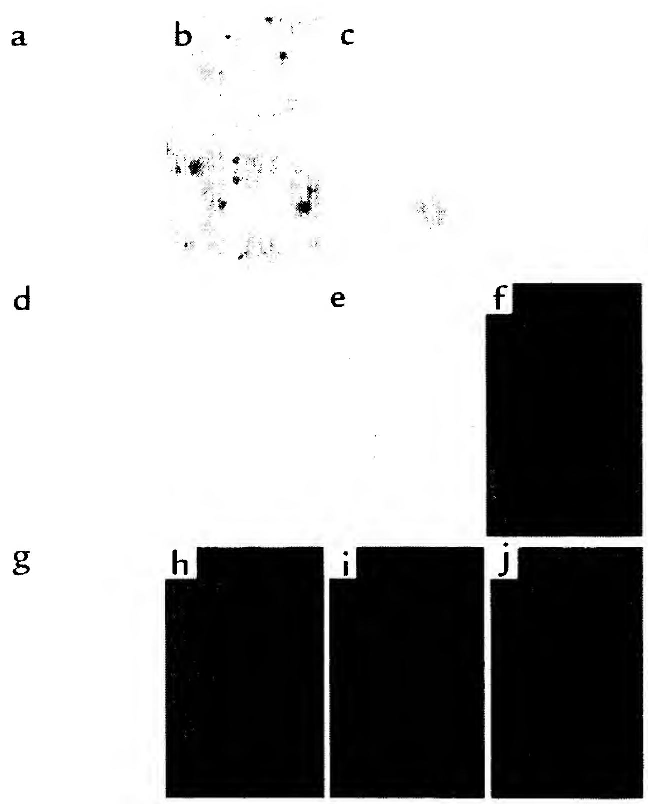


Figure 4
Incorporation of SP cells into cardiomyocytes. (**a**) Negative control: C57Bl/6 cardiac tissue stained for lacZ expression. (**b**) Positive control: C57Bl/6-Rosa26 cardiac tissue stained for lacZ expression. This typical section demonstrates both patterns of punctate and whole-fiber staining. (**c**) Cross-section of a heart from an SP cell-transplant recipient, which received an infarct. (**d**) Longitudinal section of an SP cell-transplant recipient, which received an infarct. (**e**–**h**) LacZ and α-actinin costaining of lacZ-positive fibers. (**i**) CD45 costaining of the section in **g** and **h**. (**j**) Anti-CD45 staining of spleen (positive control). Sections were stained with X-gal, and LacZ-positive sections were subsequently stained for α-actinin (**f**, **h**) and CD45 (**i**), and the sections were photographed.

Figure 5
LacZ staining occurs primarily at the border of myocardial infarction. (**a**) Lower-power (×10) photograph of mouse myocardial infarction after 4 weeks. The arrowhead points to the location of lacZ staining shown in **b** and **c**. The lighter pink tissue to the left and above the arrowhead is primarily fibrotic and results from the infarction. (**b**) Higher power (×20) photograph of the same section dual stained for lacZ and the antimacrophage Ab F480. The open arrowhead indicates a macrophage, the closed arrowhead indicates lacZ-positive cardiomyocytes (the same region shown in Figure 4, g and h). (**c**) Higher-power photograph of the same section (×40). (**d**) Macrophage density of a cardiac section after 1 hour of ischemia and 3 hours of reperfusion. The open arrowheads indicate two of the many macrophages present. The counterstain is eosin.

from the Rosa26 positive control stains in both a punctate and diffuse pattern (Figure 4b). The lacZ-positive areas costained with Ab's against α-actinin (Figure 4, e–h) and appeared to be contiguous with lacZ-negative cardiomyocytes. To be certain that the punctate staining pattern did not result from infiltrating hematopoietic cells, some sections were costained with Ab's against CD45, an antigen common to all nucleated hematopoietic cells (18). The lacZ-positive cells in Figure 4g were not positive for CD45 (Figure 4i), although the CD45 Ab brightly stained spleen sections under the same conditions (Figure 4j). Only rarely were other lacZ-positive spots positive for CD45 (not shown).

LacZ-positive myocytes appeared primarily at the edge of the myocardial scar, corresponding to the region described as being "at risk" (15). This is shown in Figure 5, where lacZ-positive myocytes can be seen in a 4-week myocardial infarction. In this section, a macrophage can also be seen in the scar by costaining (Figure 5b, open arrowhead), but the lacZ-positive cells (closed arrowhead) do not stain. At 4 weeks, the macrophages have returned to a relatively low density; thus, the lacZ staining seen in the myocyte is not explained by adhesive macrophages. Figure 5d is included as a positive control to demonstrate, using the same mAb, the macrophage density of a frozen section of a mouse heart after 1 hour of occlusion and 3 hours of reperfusion.

Table 1 shows the prevalence of lacZ-positive cells or structures in cardiac tissues from different mice. A total of four mice were examined in this experiment, three at 2 weeks after injury and one (the only surviving animal) at 4 weeks. Sections (100) from each animal were analyzed, and each positive site of lacZ staining was counted. Any staining pattern – a single blue cell, a cluster of blue cells, or an area of diffuse lacZ staining – was considered to represent one event of lacZ positivity or SP cell engraftment. The number of positive events ranged

Table 1
Prevalence of lacZ-positive cells and structures in cardiac tissues

Ischemic mouse	Week of testing	LacZ⁺ myofibers per 100 sections	% cardiomyocyte engraftment	LacZ⁺ vessels (number per total)	Mean % endothelial cell engraftment
Sham	4th	0		0	–
No. 1	2nd	243	0.02	36/1100	3.3 ± 1.9
No. 5	2nd	55	<0.01	11/600	1.8 ± 1.8
No. 6	2nd	730	0.06	39/1143	4.7 ± 2.5
No. 10	4th	45	<0.01	17/313	5.4 ± 0.9
			Mean 0.02%		Mean 3.3%

Animals were rendered ischemic by coronary artery ligation. Myofibers were counted as a single lacZ-positive structure, whether there was one blue cell, a cluster of blue cells, or a single large fiber stained throughout. The percentage of engraftment of cardiomyocytes is based on an estimate of 13,000 myocytes per section (12,929 ± 168 SD). This number was attained by photographing the total section of the mouse heart at the midventricular section used for all studies. On average, the sections contained 10.69 mm² left-ventricular area; all sections were examined with little variance. Nuclei were counted in each section. The number of cardiac nuclei varied from 12 to 1,300/mm². The average number of cardiac nuclei was taken as a reasonable estimate of the amount of cardiac myocytes visible within each section. The percentage of engraftment is based on counting 100 random sections of heart and therefore represents the engraftment for the entire cross-section, not just the area at risk. Since the peri-infarct region had a much higher prevalence of lacZ cardiomyocytes, the engraftment in the area at risk is probably larger, but the uncertainty of defining the area at risk on individual sections precluded a separate count. The vessels were quantified by determining the number of lacZ-positive vessel profiles (lacZ-positive vessel structures that were also costained with an Ab against Flt-1) per total vessels (Flt-1 positive) in the section as observed under the microscope. The mean percentage of lacZ-positive vessels is based on examination of 100 or more vessels of various sizes from 11 sections from mouse number 1, six from mouse 5, ten from mouse 6, and three from mouse 10. The vessels counted as positive had one or more endothelial cell that was lacZ positive and Flt-1 positive.

from 45 to 730 per 100 sections. Since each section contained approximately 13,000 cardiomyocytes, this represents a mean engraftment prevalence of around 0.02% of all cardiomyocytes.

To determine incorporation of SP cells into endothelial cells, approximately 100 vessel profiles were analyzed per tissue section. Three to 11 tissue sections were counted per animal. As in the cardiac tissue, a single vessel was scored as positive whether one or multiple endothelial cells per vessel profile was stained. The mean prevalence of lacZ-positive vessels was approximately 3%. This level of incorporation is somewhat lower than what has been reported for the engraftment of circulating endothelial-cell progenitors derived from whole blood (7). This is not surprising, considering that SP cells are multipotent and give rise to many cell types other than vascular endothelium. As expected, engraftment of the SP cells was predominantly into newly formed capillaries, in the "at-risk" tissue adjacent to the infarcted zone. Engraftment of SP cells into larger vessel structures was less frequent and to a lesser extent.

Discussion

Myocardial infarction is a leading cause of heart failure and death in developed countries. Although postinfarction survival rates have improved in recent years, reduced heart function due to excessive loss of cardiomyocytes remains a major problem. The lack of resident stem cells in the heart has led to an intensive search for alternative sources of cardiomyocyte progenitors. Embryonal stem cells have been shown to differentiate into cardiomyocytes that can form stable intracardiac grafts (19). Skeletal myoblasts or cardiomyocytes from fetal or neonatal mice have also been shown to take up residence in cardiac tissue after injury (20, 21). Although demonstrating the potential of cellular engraftment as a means to augment the number of myocytes in cardiac muscle, these previous studies have not identified a progenitor population readily accessible from an adult patient's own tissue that can be adapted to clinical therapy.

Here we have assessed the ability of purified stem cells derived from adult mouse bone marrow to participate in cardiac muscle regeneration following the induction of ischemia by coronary artery occlusion and reperfusion. These stem cells are a highly enriched stem cell population that can be reproducibly isolated from adult tissues. When transplanted into the bone marrow of lethally irradiated mice, purified SP cells marked with the lacZ gene regenerated the hematopoietic system. At 2 or 4 weeks after coronary artery ligation, lacZ-positive cells had migrated into cardiac myofibers, where they participated in the regeneration of healthy muscle. This level of myocardial engraftment, 0.02% of all cardiomyocytes, was similar to that seen with use of whole bone marrow or purified stem cells to regenerate skeletal muscle (2, 12). Importantly, there was no evidence of SP cell migration into the hearts of sham-operated animals. Finally, the presence of lacZ-positive hematopoietic cells in the cardiac tissue (measured with Ab's against CD45 and macrophages) was not a problem in this study, as shown in Figure 5. This is consistent with previous observations that the major inflammatory response is observed immediately after infarction and has largely resolved by 2 to 4 weeks (22).

We also found that lacZ-positive cells could participate in neovascularization in regenerating heart tissue. Many small vessels, comprising one to three endothelial cells (cell number determined by 4′,6-diamidine-2-phenylindole dihydrochloride [DAPI] staining), clearly expressed the marker gene. Some large- or medium-sized vessels also appeared to have incorporated marked cells, although such structures consisted primarily of host cells. The overall level of neovascular engraftment was approximately 3%. This is lower than the engraftment of more differentiated endothelial cell progenitors derived from whole blood (7). The majority of engraftment also appeared to be adjacent to the region of infarct. We did not attempt to determine whether these vessel structures were arterioles, venules, or lymphatic vessels.

We present evidence here that bone marrow hematopoietic stem cells, purified as SP cells, have transdifferentiated and engrafted into myocardium and endothelial cells. However, future studies can extend and confirm these conclusions using additional criteria. First, we cannot exclude the possibility that small numbers of contaminating nonhematopoietic stem cells are responsible for the cardiac and endothelial cell engraftment. Such contaminants could arise from imperfect cell sorting or could even be due to stem cells copurified by our SP isolation procedure. Clonal studies will be essential to rule out the possibility that multiple stem cell populations are present within the donor cell population. Second, current studies in the field are limited by the use of markers that are present ubiquitously in donor cells, such as lacZ, green fluorescent protein (GFP), or Y chromosome fluorescence in situ hybridization (FISH) (12, 23). While one could argue about differences in sensitivity and specificity of these marker systems, each will identify any donor cell, not just one that has differentiated into the cell type of interest. This necessitates the use of secondary criteria, such as morphology and colocalization with specific markers, to establish cell identity. Perhaps more definitive will be using donor cells that express a nuclear-localized marker gene under the control of a cell type-specific promoter. In this case, donor cells will not express the marker until they differentiate into the cell type of interest (2). Although such studies should generate less disputable data, they are also flawed in that expression of any one gene, at any given time, is not a reliable indicator of a distinct phenotype. Hence, determining donor cell localization into complex tissues in this manner will still require colocalization with other markers, as well.

Our detection of angioblast markers on purified SP cells may bear on the developmental fate of these stem

cells. Before transplantation took place, we observed no expression of genes indicative of a mature, differentiated endothelial cell phenotype in SP cells. Instead, our analyses revealed, as expected, expression of genes reported to be activated in a common progenitor of both hematopoietic cells and endothelial cells, namely PECAM-1 (24) and Tal-1 (25-27). Surprisingly, other genes thought to be specifically reflective of an embryonic endothelial cell progenitor, such as tyrosine kinase receptors Flk-1 and Flt-1 (28), were not expressed in SP cells; however, expression of their ligand VEGF-A was detected.

Perhaps expression of VEGF-A in the endogenous population of SP cells plays a role in "directing" these multipotent cells to the surface of mature endothelial cells expressing VEGF receptors in adult tissues when needed. Such a role for VEGF-A, via Flk-1-receptor signaling, has been described for angioblast migration during embryonic vascular development (29), supporting a role of VEGF-receptor binding in directing the positioning, as well as differentiation, of multipotent progenitors. Alternatively, it is also possible that VEGF-A expression by multipotent stem cells may serve a unique functional role in the stem cell microenvironment, independent of its well-documented effects on endothelial cell behavior (30), perhaps in the maintenance of a "progenitor" state

In addition to expressing VEGF-A, the bone marrow SP cells also expressed RNA for Ang-1 and its receptor, Tie-2. Ang-1 and Tie-2 are thought to be involved in an important paracrine-signaling pathway needed for the recruitment, or maintenance, of smooth muscle cells and pericytes to stabilize newly forming endothelial tubes (31). Embryos lacking either Ang-1 (31) or Tie-2 (16) die at embryonic day 11.5 and 10.5, respectively, with severe vascular malformations and hemorrhage. The role of Ang-1 and Tie-2 in the SP cells is uncertain. Since SP cells express the ligand, as well as the receptor, both autocrine and paracrine regulatory pathways can be envisioned. In a paracrine manner, perhaps Ang-1 or Tie-2 bind to their respective partner in the bone marrow stroma to modulate cell survival or responses to other soluble factors in the microenvironment, as has been proposed for mature endothelial cells (32). Certainly, much remains to be determined regarding the role of factors such as Ang-1 and VEGF-A in the modulation of SP survival, growth control, and interactions with the surrounding microenvironment.

Although endothelial cells and hematopoietic cells are thought to be related developmentally, possibly originating from a common hemangioblast precursor (33), evidence for such a cell in the adult is lacking. We suggest that the SP cells are bona fide hematopoietic stem cells with the capacity for endothelial or cardiomyocytic differentiation under circumstances of acute injury. Our findings underscore the developmental versatility of adult hematopoietic stem cells and suggest that their functional role is ultimately determined by their migration into particular microenvironments, such as myocardium, and their exposure to locally generated signals at those sites.

What cell type participates in myocardial regeneration? In our experiment, the purified stem cells stably engrafted into the bone marrow of recipient mice. The presence of lacZ-marked cells in damaged cardiac tissue could reflect direct incorporation of migrating SP cells or their progeny. Since the surface phenotypes of lacZ-positive cells that had integrated into cardiac tissues were those of differentiated myocytes and endothelial cells, we are unable to deduce the exact identity of the cells that first migrated into the heart

The incorporation of bone marrow–derived SP cells into both regenerating endothelial cells and cardiomyocytes suggests that circulating stem cells may naturally contribute to repair of these tissues. The lack of sensitive markers and similar experimental design has likely impeded previous observation. Although the contribution of our stem cells to cardiac regeneration was low improvements in efficiency that can be achieved through a better understanding of the process promise to offer new therapeutic avenues in the long term.

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PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/18, C12N 5/06, 5/08, A61K 35/34	A1	(11) International Publication Number: **WO 98/27995** (43) International Publication Date: 2 July 1998 (02.07.98)

(21) International Application Number: PCT/US97/23611

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60/033,145 20 December 1996 (20.12.96) US

(71) Applicant (*for all designated States except US*): CREATIVE BIOMOLECULES, INC. [US/US]; 45 South Street, Hopkinton, MA 01748 (US).

(72) Inventors; and
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(74) Agent: TWOMEY, Michael, J.; Testa, Hurwitz & Thibeault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TREATMENT OF MAMMALIAN MYOCARDIUM WITH MORPHOGEN LOCALLY, OR WITH MORPHOGENICALLY-TREATED MYOGENIC PRECURSOR CELLS

(57) Abstract

The present invention provides methods for the treatment, and pharmaceuticals for use in the treatment, of mammalian subjects at risk of, or afflicted with, loss of or damage to myocardial tissue. The methods involve the administration of certain morphogens, inducers of those morphogens, agonists of the corresponding morphogen receptors, or small molecule morphogenic activators, or implantation of cells induced with those agents. The morphogens useful in the invention include OP1, CBMP-2A (BMP-2), CBMP-2B (BMP-4), and other members of the morphogens family of the TGFβ superfamily of growth and differentiation factors.

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
TWOMEY, Michael, J.
Testa, Hurwitz & Thibeault, LLP
High Street Tower
125 High Street
Boston, MA 02110
ETATS-UNIS D'AMERIQUE

RECEIVED
TESTA HURWITZ & THIBEAULT
JUL 14 1998
PATENT DOCKETING

Date of mailing (day/month/year)
02 July 1998 (02.07.98)

Applicant's or agent's file reference
CRP-123PC

IMPORTANT NOTICE

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US97/23611	19 December 1997 (19.12.97)	20 December 1996 (20.12.96)

Applicant
CREATIVE BIOMOLECULES, INC. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CA,EP,JP,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   None

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   02 July 1998 (02.07.98) under No. WO 98/27995

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference CRP-123PC	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 97/23611	International filing date *(day/month/year)* 19/12/1997	(Earliest) Priority Date *(day/month/year)* 20/12/1996
Applicant CREATIVE BIOMOLECULES, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. ______
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/23611

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-14, and 16-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTE IAT[redacted]AL SEARCH REPORT

ern[redacted] Application No
PCT/US 97/23611

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 C12N5/06 C12N5/08 A61K35/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 28541 A (UNIVERSITÉ LAVAL) 19 September 1996 see the whole document ---	1-26
Y	WO 95 14079 A (INDIANA UNIVERSITY FOUNDATION) 26 May 1995 see the whole document ---	1-26
Y	YOON P D ET AL: "Myocardial regeneration: Transplanting satellite cells into damaged myocardium." TEXAS HEART INSTITUTE JOURNAL 22 (2). 1995. 119-125, XP002064605 see the whole document --- -/--	1-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1998	28. 05. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBINSON S W ET AL: "Arterial delivery of genetically labelled skeletal myoblasts to the murine heart: Long-term survival and phenotypic modification of implanted myoblasts." CELL TRANSPLANTATION 5 (1). 1996. 77-91, XP002064606 see the whole document ---	1-26
Y	CHIU R.C.J. ET AL.: "Cellular Cardiopmyoplasty: Myocardial Regeneration With Satellite Cell Implantation" ANNALS OF THORACIC SURGERY, vol. 60, no. 1, July 1995, pages 12-18, XP002064607 see the whole document ---	1-26
P,X	SCHULTHEISS T M ET AL: "A role for bone morphogenetic proteins in the induction of cardiac myogenesis." GENES & DEVELOPMENT 11 (4). 1997. 451-462, XP002064608 see the whole document ---	1-26
E	WO 98 06420 A (MCW RESEARCH FOUNDATION) 19 February 1998 see the whole document -----	1-26

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/23611

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9628541 A	19-09-1996	AU 4934496 A	02-10-1996
		CA 2215244 A	19-09-1996
		EP 0815205 A	07-01-1998
WO 9514079 A	26-05-1995	AU 1097695 A	06-06-1995
		EP 0729506 A	04-09-1996
		JP 9505471 T	03-06-1997
		US 5733727 A	31-03-1998
WO 9806420 A	19-02-1998	NONE	

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
CRP-123PC		
International application No	**International filing date** *(day/month/year)*	**Priority date** *(day/month/year)*
PCT/US97/23611	19/12/1997	20/12/1996

International Patent Classification (IPC) or national classification and IPC

A61K38/18

Applicant

CREATIVE BIOMOLECULES, INC. et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/07/1998	21. 04. 99
Name and mailing address of the international preliminary examining authority:	**Authorized officer**
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Brück, M Telephone No. (+49-89) 2399 8735

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US97/23611

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-88 as originally filed

Claims, No.:

1-30 as originally filed

Drawings, sheets:

1/13-13/13 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

Section V:

1. Reference is made to the following documents:

D1 = WO 96/28541

D2 = Annals of Thoracic Surgery, 1995, Vol. 60/1, pages 12-18

D3 = WO 92/15323*

*This document was not cited in the international search report. A copy of the document is appended hereto:

2. The present application does not meet the requirements of Article 33(2) PCT, because the subject matter of claim 20 does not appear to be novel vis-à-vis document D1.

Independent claims 20 relates to a <u>therapeutic composition</u> comprising myogenic precursor cells and a morphogen.

However, a composition comprising myogenic precursor cells and a morphogen has already been disclosed in document D1 on page 3 '...donor mouse myoblasts were grown in culture with muscular growth or trophic factors, particularly, bFGF, before transplanting them...'.

Therefore, claim 20 does not appear to be novel.

2.1 The subject-matter of claims 1-19 and 21-30 is novel because the treatment of myogenic precursor cells with morphogens, inducer of morphogens, agonists of morphogens, or small molecule morphogenic activators to promote the proliferation or differentiation into functional myocardium has not been disclosed in the prior art.

3. The present application does not meet the requirements of Article 33(3) PCT, because the subject matter of claims 1-30 does not appear to involve an inventive step vis-à-vis documents D2-D3.

Independent claims 1-4 relate, in essence, to the method for treating loss or damage of myocardium by implantation of myogenic precursor cells treated with a morphogen (claim 1), an inducer of morphogen (claim 2), an agonist of morphogen (claim 4), or a small molecule morphogenic activator (claim 4).

Independent claims 20-23 relate, in essence, to a therapeutic composition comprising myogenic precursor cells and a morphogen (claim 20), an inducer of morphogen (claim 21), an agonist of morphogen (claim 22), or a small molecule morphogenic activator (claim 23), to promote the proliferation or differentiation into functional myocardium.

Independent claims 24-27 relate, in essence, to a method of culturing myogenic precursor cells by isolating them and treating them with a morphogen (claim 24), an inducer of morphogen (claim 25), an agonist of morphogen (claim 26), or a small molecule morphogenic activator (claim 27), to promote the proliferation or differentiation into functional myocardium.

Independent claims 15, 28, 29, and 30 relate, in essence, to a method to promote the proliferation or differentiation of myogenic precursor cells into functional myocardium by treating them with a morphogen (claim 15 and 28), to the method of producing replacement cardiomyocytes by implanting the cells in claim 28 (claim 29), and to a pharmaceutical composition comprising a morphogenic agent and a mitogen (claim 30).

Document D1 describes the pretreatment of healthy donor's myoblast cultures with growth or trophic factors like bFGF on transplantation to subjects suffering of myopathy like muscular dystrophy. The recipient muscles showed a higher percentage of functional cells, demonstrated by the higher incidence of dystrophin-positive fibres.

Document D2, which represents the closest prior art, demonstrates that skeletal muscle satellite cells implanted into injured myocardium can differentiate ('milieu-influenced differentiation') into cardiac muscle fibres and thus repair damaged heart muscle (abstract).
It further describes that the cardiac environment is rich with growth factors such as bFGF, β-TGF, etc. and that these factors influence the growth and differentiation process of skeletal myoblasts *in vitro*. Further, β-TGF can change the phenotypic appearance and promote the acquisition of muscle-specific properties of cardiac fibroblasts (page 17).

Document D3 describes morphogens such as Vgr-1 (a structurally related protein of the β-TGF family) and others such as OP-1, OP-2, Vgl, etc. (pages 14-16), and their role in the regulation of cell growth and differentiation (page 3). It further describes the role of morphogens on the proliferation and differentiation in a tissue-specific manner and the induction of the progression of events that culminate in new tissue formation (page 6).

The skilled man starting from document D2 faced with the technical problem--the provision of improved cells capable of developing into functional myocardium--would combine the teachings from D2-first part (the ability of skeletal muscle satellite cells to develop into functional myocardium) with those from D3/D2-second part (the role of morphogens for the tissue-specific development/that β-TGF promotes the acquisition of muscle-specific properties of cardiac fibroblasts) and thereby arrive at the claimed invention (the treatment of myogenic precursor cells with the claimed morphogens or inducers, or activators thereof, which are a subgroup of the β-TGF superfamily as explained on page 4).
This argument holds especially in light of document D1, in which myoblasts have been treated with 'morphogens' for the development of <u>more</u> functional muscle cells.

Therefore, claims 1-4, 15, and 20-30 do not appear to be inventive vis-à-vis documents D2-D3.

Dependent claims 5-14 and 16-19 seem to contain only additional technical

features common to the art and appear also, therefore, not to be inventive.

4. For the assessment of the present claims 1-19 and 29 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may, however, allow claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Section VI:

1. The attention of the applicant is directed to the following document:

   WO 98/06420 (priority: 16.08.1996, filing: 13.08.1997, publication: 19.02.1998),

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(54) Title: BONE MORPHOGENETIC PROTEIN AND FIBROBLAST GROWTH FACTOR COMPOSITIONS AND METHODS FOR THE INDUCTION OF CARDIOGENESIS

(57) Abstract

A cardiogenic composition comprising a purified mixture of a transforming growth factor-β protein and a fibroblast growth factor is disclosed. In another embodiment, the present invention is a method of inducing cardiogenesis in cells of a non-cardiac lineage comprising the steps of exposing cells to the composition and observing the development of cardiac cells.

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BONE MORPHOGENETIC PROTEIN AND FIBROBLAST GROWTH FACTOR COMPOSITIONS AND METHODS FOR THE INDUCTION OF CARDIOGENESIS

Field of the Invention

The field of the present invention is a therapeutic composition that may be capable of inducing cardiogenesis in non-cardiac tissue. Specifically, the field of the present invention is a reagent comprising a transforming growth factor-β protein and a fibroblast growth factor protein.

Background of the Invention

Dr. John Lough's laboratory has shown that the anterior lateral plate endoderm cells from the heart forming region (HFR) of stage 6 chicken embryos is specific in its ability to induce cardiogenesis in explanted precardiac mesoderm, a process highlighted by the formation of a multilayered vesicle of rhythmically contractile cells that express sarcomeric α-actin (Sugi, Y. and Lough, J., Dev. Dyn. 200:155-162, 1994). Sugi and Lough recently reported that endoderm-associated factors, including members of the fibroblast growth factor (FGFs 1, 2 and 4) and the transforming growth factor-beta (TGF-β) families (activin A), can mimic the cardiogenic effects of HFR endoderm on precardiac mesoderm (Sugi, Y. and Lough, J., Dev. Biol. 169:567-574, 1995).

The art now lacks a composition capable of inducing cardiogenesis in non-cardiac tissue.

Disclosure of the Invention

The present invention is a cardiogenic composition comprising a purified mixture of a transforming growth factor-β protein and fibroblast growth factor. Specifically, the transforming growth factor-β protein is from the group consisting of bone morphogenetic proteins (BMPs): BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, and BMP-15. Most

preferably, the transforming growth factor-β protein is BMP-2.

Preferably, the fibroblast growth factor (FGF) is selected from the group consisting of FGFs 1 - 15. Most preferably, the fibroblast growth factor is either FGF-4 or FGF-2.

In another embodiment, the present invention is a method for inducing cardiogenesis in cells of a non-cardiac lineage comprising the steps of exposing cells to a purified mixture of bone morphogenetic protein-2 and a fibroblast growth factor (FGF-2 or FGF-4) and observing the development of rhythmical contractile cells expressing sarcomeric α-actin. The exposure may be either *in vitro* or *in vivo*.

In one embodiment of the present invention, the protein mixture is applied exogenously to the cells *in vitro*. In another embodiment of the present invention, the cells are transformed with genetic constructs encoding bone morphogenetic protein and fibroblast growth factor. The genetic constructs are then allowed to express the cardiogenic proteins.

It is an important feature of the present invention that cardiac cells can be induced from non-cardiac tissue.

Other objects, features and advantages of the present invention will become apparent after examination of the specification, drawing and claims.

Brief Description of the Drawings

Fig. 1A and B describe the incidence of cardiogenesis in non-precardiac mesoderm cells treated with BMP-2 and FGF-4. Fig. 1A diagrams the heart-forming region (pre-cardiac tissue) and the non-precardiac tissue region of a stage 6 avian embryo. Fig. 1B is a graph of percent contractile explants that are obtained, versus treatment with FGF-4, BMP-2, or FGF-4 and BMP-2 combined.

Best Modes for Carrying out the Invention

The present invention is a composition and method for the induction of cardiogenesis in non-precardiac tissue, preferably human tissue. By "cardiogenesis" we mean the development of rhythmically and synchronously contractile cells that express sarcomeric α-actin from cells that are not part of the cardiac lineage. Preferably, a cell can be identified as a cardiac cell by visual observation via microscopy. The Examples below demonstrate that a monoclonal antibody that recognizes sarcomeric α-actin can confirm that cells are expressing α-actin.

By "non-precardiac tissue," we mean tissue that is not part of the cardiac lineage. For example, the following tissues are non-precardiac: fibroblasts, which make connective tissue, and cells that will become other mesoderm derivatives, such as skeletal muscle.

In one embodiment, the composition comprises a member of the bone morphogenetic protein subfamily of the transforming growth factor-β (TGF-β) family and a fibroblast growth factor (FGF). Preferably, the reagent comprises a mixture of bone morphogenetic protein-2 (BMP-2) and FGF-4.

The composition comprises a "purified" mixture of proteins. By "purified" we mean that the proteins in question has been purified from native or recombinant bacterial sources. For example, a crude cell extract is "purified" as is a combination of proteins that have been individually purified to almost 100% homogeneity.

The experiments described below in the Example section demonstrate that combined BMP-2 and FGF-4, but neither factor alone, induce cardiogenesis in non-precardiac avian embryonic mesoderm. We envision that this combination of protein molecules, and perhaps other combinations of TGF-β and FGF family members, will induce

cardiogenesis in other non-precardiac tissues, such as human non-cardiac tissue.

A typical source for BMP-2 is bone or recombinant human BMP-2 that is expressed in bacteria.

In addition to or in place of BMP-2, the compositions of the present invention may comprise one or more other proteins which are members of the TGF-β superfamily of proteins, particularly those which maintain the highly conserved 7-cysteine structure which is characteristic of the bone morphogenetic protein subfamily which includes BMP-2. Thus, the compositions of the present invention may include therapeutically useful agents such as the BMP proteins BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7, disclosed, for instance, in United States Patents 5,013,649; 5,116,738; 5,106,748; 5,187,076; and 5,141,905; BMP-8, disclosed in PCT publication WO 91/18098; and BMP-9, disclosed in PCT publication WO 93/00432; BMP-10, disclosed in PCT application WO 94/26893; BMP-11, disclosed in PCT application WO 94/26892; BMP-12 or BMP-13, disclosed in PCT application WO 95/16035; or BMP-15, disclosed in co-pending patent application, Serial No. 08/446,924, filed on May 18, 1995.

Other compositions which may also be useful include Vgr-2, described in Jones, et al., Mol. Endocrinol. 6:1961-1968 (1992), and any of the growth and differentiation factors GDFs, including those described in PCT applications WO 94/15965; WO 94/15949; WO 95/01801; WO 95/01802; WO 94/21681; WO 94/15966; and others.

Also useful in the present invention may be BIP, disclosed in WO 94/01557; and MP52, disclosed in PCT application WO 93/16099.

A preferred source for FGF-4 is from recombinant bacteria that express the human protein.

In addition to or in place of FGF-4, which is also known as k-FGF and is described in United States patent

5,430,019, the compositions of the present invention may comprise one or more other proteins which are members of the FGF family of proteins, such as basic FGF (FGF-2), acidic FGF (FGF-1), and other FGFs which are also described in U.S. patent 5,430,019. The disclosures of all of the above-identified applications, patents and articles are hereby incorporated by reference. Preferably, the fibroblast growth factor is selected from the group consisting of FGFs 1 - 15. Most preferably, the FGF is either FGF-2 or FGF-4.

The compositions of the invention may comprise, in addition to a TGF-β protein and an FGF protein, other therapeutically useful agents, including growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF-α and TGF-β), activins, inhibins, and insulin-like growth factor (IGF).

If one wished to determine whether a composition was suitable for the present invention, one could evaluate a test compound by the method described below to evaluate avian non-precardiac mesoderm. A suitable compound of the present invention would induce cardiogenesis in test avian non-precardiac mesoderm cells to a degree of at least 50% of that demonstrated below.

The compositions of the present invention may also include an appropriate matrix. For instance, one might desire a matrix for supporting the composition and providing a surface for growth of cardiomyocytes and/or other tissue growth. The matrix may provide slow release of the protein and/or the appropriate environment for presentation thereof and an appropriate environment for cellular infiltration. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will

define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined polymers, such as polymers of polylactic acid, polyglycolic polyorthoesters and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as collagen. Further matrices comprise pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may comprise combinations of any of the above mentioned materials and other suitable types of material and may be altered in composition and processing to alter pore size, particle size, particle shape, and biodegradability.

Preferably, the bone morphogenetic protein and the fibroblast growth factor protein will be mixed in a 1:1 molar ratio. By "1:1" we mean a variation of at least 20% is still permissible. However, other ratios may result in cardiogenesis and may also be suitable.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the protein composition, e.g. amount of tissue desired to be formed, the site of tissue damage, the condition of the damaged tissue, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of proteins in the composition. The addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also affect the dosage.

Potential uses of the compositions of the present compositions include use of the composition to treat patients with cardiac tissue damage or stress. The composition may be used as an adjunct to surgical

procedures in which the composition may be applied directly to damaged or stressed tissue. In this embodiment, the composition may be used by itself or in conjunction with cultured cells which are capable of differentiation into cells of cardio- or cardiomyocyte lineage. Among the cells which may be useful in such an embodiment are cultured cardio- or cardiomyocytes, precursor cells to cardio- or cardiomyocyte lineage, fibroblasts, embryonic mesodermal cells, or earlier embryonic stem cells which are capable of differentiating into mesoderm.

Alternatively, the composition may be used to treat cells, whether autologous or heterologous, to promote the growth, proliferation, differentiation and/or maintenance of cells of a cardio- or cardiomyocyte lineage. The cells thus treated may then be applied to the damaged or stressed tissue, either alone or in conjunction with the protein composition of the present invention.

In another embodiment, DNA sequences encoding the proteins of the present compositions may be transfected into cells, rendering the cells capable of producing the BMP and FGF proteins. The transfected cells, which are capable of producing the BMP and FGF proteins, may then be implanted at the site of damaged or stressed tissue.

An appropriate matrix may be used with any of the above embodiments in order to maintain the composition and/or cells at the site of damaged or stressed tissue. Alternatively, an injectable formulation of the composition may be used for administration of the compositions of protein and/or cells. The above may also be used for prophylactic measure in order to prevent or reduce damage or stress to tissue.

Examples

1. In General

Because immunostaining to detect additional TGF-β family growth factors in HFR endoderm revealed a provocative expression pattern for *Drosophila* decapentaplegic (dpp)-like proteins, we performed a degenerate reverse transcription/polymerase chain reaction (RT/PCR) screen to identify vertebrate dpp-like factors that are expressed by these cells. Among more than 50 PCR products sequenced to date, over half are identical to bone morphogenetic factor-2 (BMP-2).

We then investigated whether BMP-2 mimics the cardiogenic effects of HFR endoderm on precardiac mesoderm, as well as its ability to re-specify non-precardiac mesoderm to the cardiac lineage. We report here that, when present as the only supplement in defined medium, BMP-2 cannot support viability of either precardiac or non-precardiac mesoderm. Although FGF-4 can support cardiogenesis in precardiac mesoderm, this factor did not induce cardiogenesis in non-precardiac mesoderm, although explant growth was maintained. Remarkably, however, treatment of non-precardiac mesoderm with combined FGF-4 and BMP-2 induced cardiogenesis in a high incidence of explants, indicating that this combination of growth factors is able to re-specify embryonic cells to the cardiac lineage.

2. Materials and Methods

Explantation and Culture of Embryonic Mesoderm:

Chicken embryos were staged according to the criteria of Hamburger and Hamilton (Hamburger, V. and H. L. Hamilton, J. Morphol. 38:49-92, 1951). Anterior lateral plate precardiac mesoderm, and non-precardiac mesoderm from the posterior half of stage 6 embryos, was micro-dissected, explanted to Lab-Tek chamber slides and cultured in M199 as previously described (Sugi, Y. and J.

Lough, supra, 1994; Sugi, Y. and J. Lough, supra, 1995). Growth factors were added to the indicated final concentrations after explants attached to the fibronectin substrate. Human recombinant FGF-4 was purchased from R&D Systems. Human recombinant BMP-2 was provided by the Genetics Institute (Cambridge, MA). Medium, including growth factors, was changed daily.

Immunohistochemistry:

Biochemical differentiation was monitored by immunohistochemistry, using a monoclonal antibody that recognizes sarcomeric α-actin (Sigma, Cat. No. A-2172); the secondary antibody was fluorescent isothiocyanate (FITC)-labeled goat anti-mouse IgM (Cappel). Decapentaplegic-like protein was localized using a polyclonal antibody (1:1,000) provided by Dr. F. Michael Hoffmann (University of Wisconsin; Panganiban, G.E.F., et al., Mol. Cell. Biol. 10:2669-2677, 1990) that recognizes *Drosophila* decapentaplegic. Controls consisted of identically diluted normal rabbit serum which was used as the primary antibody, and omission of the primary antibody. The secondary antibody was FITC-conjugated goat anti-rabbit IgG (1:500). All immunohistochemical procedures, including determinations of 5'-bromodeoxyuridine incorporation, have been previously described (Sugi, Y. and J. Lough, supra, 1995).

Reverse Transcription-Polymerase Chain Reaction (RT/PCR):

RNA from microdissected stage 6 HFR endoderm was purified, with 5 μg linear polyacrylamide as carrier, using RNAstat (Tel-Test, Inc.). Complementary DNA was synthesized by M-MLV reverse transcriptase-mediated extension of oligo-dT-primed RNA. To ensure the absence of contaminating genomic DNA, non-reverse transcribed RNA was simultaneously processed. Degenerate primers were designed to recognize conserved domains in the TGF-β dpp subfamily. The upstream primer was 5'-TGGAATTCGGITGGVAIGAYTGGAT-3' (96-fold degenerate) (SEQ ID NO:1); the reverse complement of the downstream target

was 5'-GAGGATCCGGIACRCARCAIGCYTT-3' (128-fold degenerate) (SEQ ID NO:2).

Complementary DNAs were amplified using *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) with 40 cycles of denaturation (94°C, 1 minute), primer annealing (45°C, 1.5 minutes) and extension (72°C, 2 minutes). Complementary DNAs in the predicted 200 bp product were cloned into pCRScript (Stratagene). Identity of cloned inserts was determined by sequencing and comparison with the GenBank/EMBO database.

3. <u>Results</u>

Immunohistochemical Localization of DPP-Like Protein in HFR Endoderm:

To ascertain whether *Drosophila* decapentaplegic (dpp)-like proteins were associated with HFR endoderm, the anti-dpp immunostaining pattern of cultured HFR endoderm was determined in comparison with explanted precardiac mesoderm. The periphery of HFR endoderm cells exhibited intense staining, which was not observed in precardiac mesoderm or in control explants stained with normal rabbit serum.

RT/PCR Demonstration of BMP-2 in HFR Endoderm:

Because dpp is 75% homologous with BMPs 2 and 4, it was considered that the antigens described above represented these factors or perhaps other members of the TGF-β dpp subgroup. To identify dpp mRNAs that are expressed by HFR endoderm, a RT/PCR screen was performed using degenerate primers targeted to conserved domains in this subgroup. A single 200 bp PCR product, which was the predicted size for dpp cDNAs, was cloned and sequenced to identify individual dpp-like factors that are expressed by HFR endoderm. Among approximately 50 cDNAs sequenced to date, more than half were identical to BMP-2 over a 162 base stretch corresponding to nucleotides 800-962 of the chicken homologue (Francis, P.H., <u>et al</u>., <u>Development</u> 120:209-218, 1994), a domain specified by the primers. No other known or novel member

of the dpp subgroup has been identified. Although the remaining, cloned cDNAs exhibited sequences that were similar to each other, these are not homologous to any database entries. These findings suggest that BMP-2 is the major member of the dpp group that is expressed by HFR endoderm.

BMP-2 & FGF-4 Induce Cardiogenesis in Non-Precardiac Mesoderm:

Based on these results, it was of interest to determine whether BMP-2, when present alone in defined medium, could emulate the cardiogenic effect of HFR endoderm on precardiac mesoderm. Unlike FGFs (Sugi, Y. and J. Lough, supra, 1995; Zhu, X., et al., "Evidence for fibroblast growth factor 1 and 4 participation in paracrine and autocrine mechanisms that regulate embryonic heart development," submitted), BMP-2 supported neither survival nor differentiation of precardiac mesoderm. However, as anticipated, inclusion of FGF-4 with BMP-2 triggered terminal cardiogenesis in precardiac mesoderm.

Fig. 1A and B describe the incidence of cardiogenesis in non-precardiac mesoderm treated with BMP-2 and FGF-4. Non-precardiac mesoderm was explanted from the posterior half of stage 6 embryos and cultured in the presence of FGF-4 and/or BMP-2 at the indicated concentrations. Whereas neither FGF-4 nor BMP-2 alone induced cardiogenic differentiation in any explant, the majority of explants treated with both growth factors exhibited cellular multilayering and rhythmic contractility within 1 or 2 days. Referring to Fig. 1B, numbers in parentheses indicate experimental repetitions that were conducted during the aggregate of five experiments, each of which included approximately five replicate explants; the incidence of differentiation (40-60%) was similar within each experimental repetition.

As diagramed in Fig. 1A, these determinations were performed on non-precardiac mesoderm explanted from the posterior region of stage 6 embryos. Cells in this area

are destined to become extraembryonic mesoderm and lateral plate mesoderm that is not cardiogenic (review: Nicolet, G., Adv. Morphogenesis 9:231-262, 1971). As shown in Fig. 1B, neither FGF-4 nor BMP-2 alone could induce formation of contractile explants in non-precardiac mesoderm. Cells cultured with BMP-2 alone detached from the culture dish and did not survive; and, although treatment with FGF-4 alone supported cellular proliferation as evidenced by 5'-bromodeoxyuridine incorporation, differentiation was never observed. Remarkably however, the combined presence of FGF-4 and BMP-2 caused cardiogenic differentiation in over half of the non-precardiac mesoderm explants (Fig. 1B), as indicated by formation of a multicellular vesicle which exhibited rhythmic contractility and sarcomeric α-actin differentiation. Because differentiation of non-precardiac mesoderm was not usually observed until the second day *in vitro* (Fig. 1B), in contrast to precardiac mesoderm in which differentiation is observed on day one, the occurrence of a re-specification step in non-precardiac mesoderm explants is suggested. These findings indicate that these growth factors synergistically function to induce cardiogenesis in cells that are not fated to the cardiac lineage.

4. Discussion

In addition to verifying our previous findings that HFR endoderm is specific in its ability to induce terminal differentiation (Sugi, Y. and J. Lough, supra, 1994), Schultheiss, et al. (Schultheiss, T.M., et al., Development 121:4203-4214, 1995) also reported that HFR endoderm is capable of re-specifying embryonic cells to the cardiogenic lineage. Regarding the latter, our observation that HFR endoderm was unable to re-specify posterior non-precardiac mesoderm (Sugi, Y. and J. Lough, supra, 1994) apparently reflected inadequate local concentrations of endodermal factors, caused by placing

the explants at opposite sides of the culture dish rather than in contiguity. The findings reported herein that the combined activities of BMP-2 and FGF-4, both of which are expressed in HFR endoderm, induce cardiogenesis in non-precardiac mesoderm are consistent with the ability of HFR endoderm to re-specify cells to the cardiogenic lineage.

We previously determined that HFR endoderm expresses three members of the FGF family (FGFs 1, 2 and 4), each of which supports differentiation of precardiac mesoderm (Sugi, Y. and J. Lough, supra, 1995; Zhu, X., et al., supra, in press, Developmental Dynamics). Moreover, experiments utilizing sodium chlorate to specifically prevent binding of FGF to cognate receptors have indicated that FGF signaling is obligatory for endoderm-mediated cardiogenesis (unpublished observations). However, because the major role of FGF is apparently to regulate precardiac mesoderm proliferation, cooperation with "differentiation" factors such as BMP-2 may be required to induce cardiac myocyte differentiation. In this interpretation, our observations that isolated precardiac mesoderm can differentiate in culture with FGF alone suggests that these cells had been signaled by BMP-2 prior to explantation from the embryo. Although cooperativity between activin and FGFs has been shown to regulate mesoderm induction (Cornell, R. and D. Kimelman, Development 120:453-462, 1994; LaBonne C. and M. Whitman, Development 120:463-472, 1994) in *Xenopus*, our preliminary findings indicate that activin cannot replace BMP-2 in supporting the differentiation of non-precardiac mesoderm. And, although FGF-4 and BMP-2 are expressed in the apical ectodermal ridge of the mouse limb bud to respectively stimulate and inhibit limb growth (Niswander L. and G.R. Martin, Nature 361:68-71, 1993), BMP-2's inhibitory effect on growth could be considered as a consequence of its differentiative function. Finally, the possibility that the BMPs have differentiative

potency has recently been reported by Pourquie, et al. (1995) who demonstrated that BMP-4 specifies myotome cells to the skeletal myocyte lineage (Pourquie, O., et al., Cell 84:461-472, 1995).

5. Prophetic Results Of Animal Experiments

It is anticipated that ongoing experiments using a rabbit model will reveal that the combination of bone morphogenetic protein (BMP) and fibroblast growth factor (FGF), as opposed to either factor alone, will significantly augment protection of the heart (cardioprotection) from an experimentally-induced transient episode of ischemia and reperfusion.

Rationale: The rationale for these experiments is as follows. Basic fibroblast growth factor (bFGF; FGF-2) alone has been shown to increase functional recovery in isolated rats heart when given immediately prior to induction of ischemia and reperfusion (Padua, R.R., et al., Mol. Cell Biochem. 143:129-135, 1995). This cardioprotective effect was achieved using a dose of 10 μg FGF-2 per rat heart, administered directly into the coronary vasculature. In dogs, moreover, FGF-2 treatment has recently been shown to reduce the size of myocardial infarctions, 7 days after reperfusion without hemodynamic effects or evidence of neovascularization; this cardioprotective effect was achieved when FGF-2 was given intracoronary at a dose of 10 μg per dog heart prior to reperfusion (Horrigan, M.C.G., et al., Circulation 94:1927-1933, 1996).

Osteogenic protein-1 (hOP-1) is a member of the TGF-β superfamily that is closely related to BMP. hOP-1 given intravenously prior to reperfusion at a dose of 20 μg/animal reduced reperfusion injury 24 hours later in an *in vivo* rat model of coronary artery ischemia and reperfusion (Lefer, A.M., et al., J. Mol. Cell Cardiol. 24:585-593, 1992).

FGF-4 + BMP-2: We recently discovered that combined FGF-4, or FGF-2, plus BMP-2 induces embryonic mesoderm cells that are not destined to the cardiogenic lineage to differentiate into a multilayered, synchronously contractile hollow vesicle that resembles a microscopic heart *in vitro;* treatment with FGF-4 (or FGF-2) alone, or BMP-2 alone, had not effect (Lough, J., et al., Developmental Biology 178:198-202, 1996). From this result we predict that BMP functions in the embryo to recruit cells to the cardiogenic lineage, and that FGF is required to support these cells along the cardiac differentiative pathway. Unlike the situation in the embryo, cells in the adult heart neither grow nor develop; as a result, damage suffered during insults such as myocardial infarction is frequently catastrophic. The reason(s) adult cardiac myocytes are unable to undergo growth and regeneration is unknown. It is our expectation that the unavailability of growth factors such as FGF and BMP in a functionally useful context is a major cause for the inability of cells in the adult heart to regenerate.

Experimental Plan: General

Regarding cardioprotection, we are unaware of any studies examining the role of BMP *per se*, either alone or in conjunction with other growth factors, in maintaining cardiac function or protecting the myocardium from the effect of ischemia and reperfusion, a situation that is encountered in patients undergoing elective ishemia, such as heat bypass surgery and angioplasty, and heart attacks. It is our expectation that, by combining BMP-2 and FGF-2, the cardioprotective effect described above for FGF-2 alone will be augmented. Accordingly, we are determining the physiological advantage of treatment with combined growth factors on pre-ischemic aerobic cardiac function and post-ischemic recovery following ischemia, using a protocol which is routinely used in the

laboratory of one of the inventors, Dr. John Baker. The experimental model utilizes the isolated rabbit heart, which is subjected to one cycle of ischemia and reperfusion. Recovery of heart function from the ischemia/reperfusion insult will be used as an endpoint to determine the effectiveness of the combined growth factor treatment. The basis of this cardioprotective effect will be determined at the cellular and molecular level in terms of whether renewed cardiac myocyte growth and differentiation occur, using techniques that are routine in Dr. Lough's laboratory.

Experimental Plan: Specific

Experiments will be performed in a random manner using four groups to determine whether BMP-2 synergistically improves the cardioprotective effect of FGF-2. The four experimental groups are as follows:
Group 1, saline vehicle control (no growth factors) - I.V. route of administration
Group 2, BMP-2 (80 μg/kg i.v.) alone
Group 3, FGF-2 (40 μg/kg i.v.) alone
Group 4, BMP-2 (80 μg/kg i.v.) in combination with FGF-2 (40 μg/kg i.v.)

Dosages and intended routes of administration are based on published reports that have demonstrated cardioprotective effects of FGF and BMP-related growth factors (1-3). Each group will consist of eight (8) immature rabbits, 7-10 days old; by this neonatal stage of development, the heart is fully functional, consisting of cardiac myocytes which have recently ceased their normal proliferation. For all groups, treatments will be initiated 24 hours prior to heart excision and study to allow time for these agents to exert a (presumptively) prophylactic effect on the heart. Twenty-four hours after treatment the aorta will be cannulated and perfusion of the isolated heart will be immediately commenced with bicarbonate buffer at 39°C in the

Langendorff mode at a constant pressure. Saline-filled latex balloons will be placed in the left and right ventricles for measurement of developed pressures. Hearts will be perfused for a 30 minute equilibration period during which time biventricular function and coronary flow rate will be recorded under steady-state conditions. Hearts will be subjected to a 30 minute period of global, no-flow ischemia at 39°C. After the ischemic period, heart function will again be measured under steady-state conditions. Thus, each heart serves as its own control. Recovery of cardiac function will be expressed as a percentage of its pre-ischemic value. The results will be evaluated by comparing pre-ischemic and post-ischemic function within the growth factor-treated groups (Groups 2-4) with recovery in the control group (Group 1).

Industrial Applicability

The present invention is a therapeutic composition capable of inducing cardiogenesis in non-cardiac tissue. The composition is a purified mixture of transforming growth factor β-protein and fibroblast growth factor.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MCW Research Foundation, Inc.
(B) STREET: 8701 Watertown Plank Road
(C) CITY: Milwaukee
(D) STATE: Wisconsin
(E) COUNTRY: United States of America
(F) POSTAL CODE: 53226
(G) TELEPHONE: (414) 456-4402
(H) TELEFAX: (414) 266-8522

(ii) TITLE OF INVENTION: CARDIOGENESIS COMPOSITION

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Quarles & Brady
(B) STREET: 411 East Wisconsin Avenue
(C) CITY: Milwaukee
(D) STATE: Wisconsin
(E) COUNTRY: U.S.A.
(F) ZIP: 53202-4497

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Baker, Jean C.
(B) REGISTRATION NUMBER: 35,433
(C) REFERENCE/DOCKET NUMBER: 650053.91134

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (414) 277-5709
(B) TELEFAX: (414) 271-3552

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(11..12, "")
(D) OTHER INFORMATION: /note= "Position 11 is listed as N but was inosine."

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(17..18, "")
(D) OTHER INFORMATION: /note= "Position 17 is listed as N but was inosine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGAATTCGG NTGGVANGAY TGGAT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(11..12, "")
(D) OTHER INFORMATION: /note= "Position 11 is listed as N but was inosine."

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(20..21, "")
(D) OTHER INFORMATION: /note= "Position 20 is listed as N but was inosine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAGGATCCGG NACRCARCAN GCYTT 25

CLAIMS

We claim:

1. A cardiogenic composition comprising a purified mixture of a transforming growth factor-β protein and a fibroblast growth factor.

2. The composition of claim 1 wherein the transforming growth factor-β protein is selected from the group consisting of BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, and BMP-15.

3. The composition of claim 2 wherein the transforming growth factor-β from the group consisting of members of the 7-cys structure family.

4. The composition of claim 3 wherein the transforming growth factor-β protein is BMP-2.

5. The composition of claim 1 wherein the fibroblast growth factor is selected from the group consisting of FGFs 1 - 15.

6. The composition of claim 1 wherein the fibroblast growth factor is FGF-4.

7. The composition of claim 1 wherein the ratio of transforming growth factor-β protein and fibroblast growth factor is a 1:1 molar ratio.

8. The composition of claim 1 wherein the composition additionally comprises a matrix material.

9. The composition of claim 1 wherein the matrix material is collagen.

10. A method for inducing cardiogenesis in cells of a non-cardiac lineage comprising the steps of

a. exposing cells to a purified mixture of a transforming growth factor-β protein and a fibroblast growth factor, and

b. observing the development of rhythmic, synchronously contractile cells expressing sarcomeric α-actin.

11. The method of claim 10 wherein the mixture of transforming growth factor-β protein and fibroblast growth factor is achieved by exogenously applying a mixture of the proteins to the cells.

12. The method of claim 10 wherein the exposure is achieved by transforming the cells with a genetic construct encoding transforming growth factor-β protein and fibroblast growth factor.

13. The method of claim 10 wherein the exposure is *in vivo*.

14. The method of claim 10 wherein the exposure is *in vitro*.

FIG. 1A

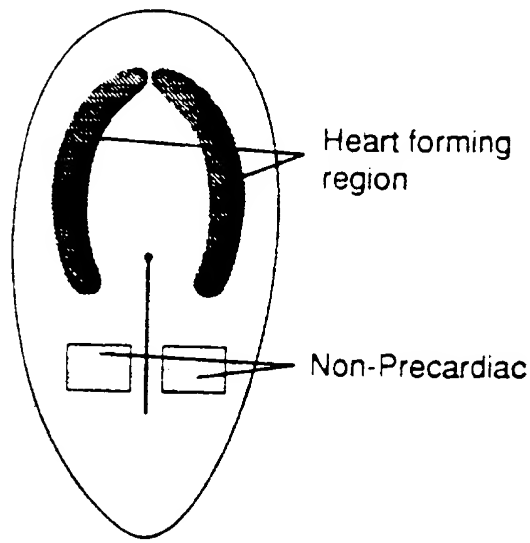
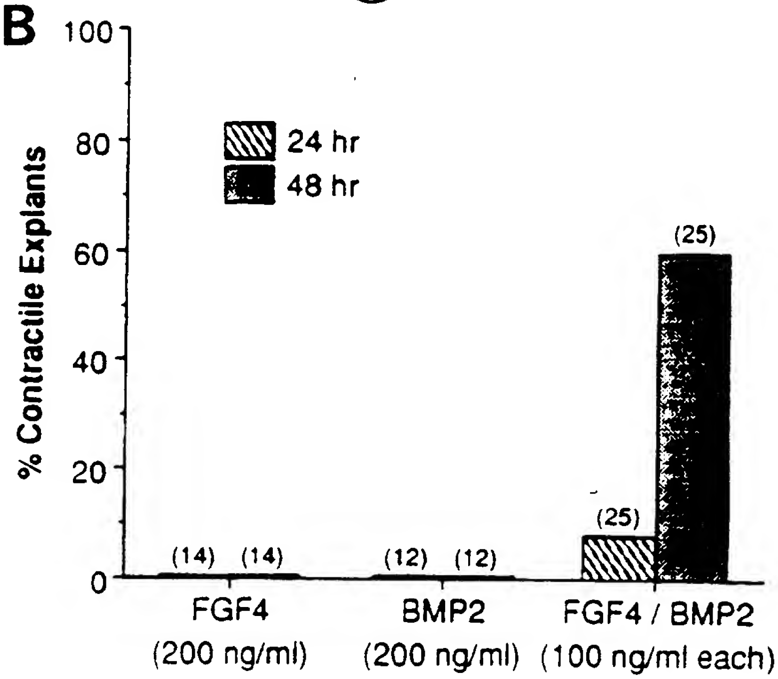


FIG. 1B



BMP-2 + FGF-4 Induce Cardiogenesis in Non-Precardiac Mesoderm

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14229

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/18, 48/00; C12P 21/00; C12N 5/10, 5/06
US CL : 424/198.1; 435/69.1; 514/12, 44; 435/375, 384
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1; 435/69.1; 514/12; 435/375, 384

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NISWANDER et al. FGF-4 and BMP-2 have opposite effects on limb growth. NATURE. 07 January 1993, Vol. 361, pages 68-71, especially page 69.	1-7
Y	US 5,013,649 A (WANG et al) 07 May 1991, see entire document, especially column 5, line 55 to column 6, line 24.	8-9
Y	MIMA et al. Fibroblast growth factor receptor is required for in vivo cardiac myocyte proliferation at early embryonic stages of heart development. Proc. Natl. Acad. Sci. USA. January 1995, Vol. 92, pages 467-471, especially pages 468-471.	10-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 SEPTEMBER 1997	29 OCT 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Deborah Freese for David S. Romeo
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUGI et al. Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis in vitro. Developmental Biology. 1995, Vol. 168, pages 567-574, especially pages 569-573.	11-14
Y	SUGI et al. Inhibition of precardiac mesoderm cell pr..iferation by antisense oligodeoxynucleotide complementary to fibroblast growth factor-2 (FGF-2). Developmental Biology. 1993, Vol. 157, pages 28-37, especially pages 31-36.	11-14
A,P	SASAI et al. Ectodermal patterning in vertebrate embryos. Developmental Biology. 01 February 1997, Vol. 182, pages 5-20, see entire document.	1-14
A,P	LOUGH et al. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. Developmental Biology. 25 August 1996, Vol. 178, pages 198-202.	1-14
A,P	NEUBUSER et al. Antagonistic interactionsbetween FGF and BMP siganlling pathways: a mechanism for positioning the sites of tooth formation. Cell. 25 July 1997, Vol. 90, pages 247-255.	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14229

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest
☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS
search terms: composition, combination, mixture, bone morphogenetic protein, bmp, fibroblast growth factor, fgf, transforming growth factor, tgf, cardiogenesis, cardiogenic, cardiac, sarcomeric

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-9, drawn to compositions comprising a transforming growth factor-beta protein and a fibroblast growth factor.
Group II, claim(s) 10, 11, 13 and 14, drawn to methods of administering a composition comprising transforming growth factor-beta and fibroblast growth factor.
Group III, claim(s) 10, 12, 13 and 14, drawn to methods of transforming cells with a construct encoding a transforming growth factor-beta and a fibroblast growth factor.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: PCT Rule 13.2 states that unity of invention exist only when there is a technical relationship among the claimed inventions involving one or more special technical features. The term "special technical feature" is defined as meaning those technical features that define a contribution which each of the inventions, considered as a whole, makes over the prior art. The special technical feature of each of Groups I-III is a mixture of a transforming growth factor-beta and a fibroblast growth factor. However, compositions comprising a transforming growth factor-beta and a fibroblast growth factor and the effects of these compositions on cell growth and differentiation are known in the art. See NISWANDER et al. Nature (07 January 1993), Vol. 361, pages 68-71. Therefore the inventions of Groups I-III do not fulfill the requirements for unity of invention with respect to the claimed compositions and the claimed methods of administering the mixture of a transforming growth factor-beta and a fibroblast growth factor.